HIV's Interactions with the Intestinal Mucosa Determine the Disease Course

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Dissertation

zur
Erlangung der naturwissenschaftlichen Doktorwürde
(Dr. sc. nat.)
vorgelegt der
Mathematisch-naturwissenschaftlichen Fakultät
der
Universität Zürich

von

Ursula Hofer
von Schüpfen, BE

Promotionskomitee

Prof. Dr. Roger M. Nitsch (Vorsitz)
Prof. Dr. Roberto F. Speck (Leitung der Dissertation)
Prof. Dr. Adriano Aguzzi
Prof. Dr. Thomas Brunner
Prof. Dr. Nicola Harris

Zürich, 2010

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Summary

Despite major efforts in prevention and treatment, the HIV epidemic remains a global problem affecting millions of people. Understanding the basic mechanisms governing HIV transmission and pathogenesis is a prerequisite to develop new therapeutic strategies.

One of the organs most affected by HIV is the gut. The intestinal mucosa is densely populated with CD4+ T-cells and macrophages, the main HIV target cells. The aim of this thesis was to define the role of the gut for HIV transmission and pathogenesis *in vivo*. To this end, we used mice reconstituted with human, hematopoietic stem cells, so called humanized mice. Humanized mice develop a lymphoid system of human origin, and therefore, can be infected with HIV, a human-specific virus.

First, we evaluated the potential of humanized mice as a model for studying rectal HIV transmission. Injection of HIV reliably led to infection of mice, but rectal challenge rarely initiated HIV infection. In contrast to most other lymphoid organs in the mice, the gut contained almost no human cells. This disproportionately low intestinal engraftment probably is the reason for the low HIV transmission rates we observed. Even when we increased the HIV infection risk, for example by inducing colitis, rectal HIV transmission was infrequent. Thus, humanized mice are unsuitable for investigating rectal HIV transmission.

The putative disadvantage of lacking intestinal engraftment though enabled us to elucidate a key mechanism of HIV pathogenesis. In chronically HIV-infected patients, depletion of intestinal CD4+ T-cells and increased intestinal permeability is correlated with disease progression. Comparing uninfected and HIV-infected mice with and without intestinal barrier dysfunction, we identified underlying mechanisms linking intestinal barrier function and HIV pathogenesis. HIV infection induced translocation of intestinal bacteria to systemic compartments. Uninfected animals controlled this translocation by increasing the phagocytic capacity of macrophages. In HIV-infected mice, macrophage phagocytosis was inadequate leading to uncontrolled translocation, activation of T-cells, increased HIV replication and T-cell loss. Macrophage dysfunction seems to determine HIV pathogenesis.

In conclusion, our results underscore the importance of the gut as a HIV target organ. Intestinal CD4+ cells are a prerequisite for rectal HIV transmission; after mucosal HIV exposure systemic infection is not possible without local spreading. In chronic infection, disturbed intestinal barrier function has negative consequences for the whole body. Modulating the control of bacterial translocation might be a therapeutic approach.

Zusammenfassung

Die globale HIV Epidemie bleibt trotz ständiger Verbesserung von Therapie und Prävention ein schwerwiegendes Problem, das Millionen von Menschen betrifft. Das Verständnis von HIV-Transmission und Pathogenese ist eine Grundvoraussetzung um neue therapeutische Ansätze zu finden.

Eines der am meisten von HIV beeinträchtigten Organe ist der Darm. Die intestinale Schleimhaut ist dicht gepackt mit CD4+ T-Zellen und Makrophagen, den Hauptzielzellen von HIV. In der aktuellen Arbeit charakterisierten wir die Rolle des Darmes für die HIV Infektion. Dazu benutzten wir humanisierte Mäuse als *in vivo* HIV-Modell. Normale Mäuse sind resistent gegenüber HIV, humanisierte Mäuse hingegen haben dank einer Stammzelltransplantation humane Immunzellen und können mit HIV infiziert werden.

Zuerst evaluierten wir die Eignung humanisierter Mäuse als Modell rektaler HIV-Transmission. Die Mäuse konnten zuverlässig infiziert werden, wenn wir HIV injizierten, rektale Exposition jedoch führte selten zur Infektion. Der Darm enthielt fast keine humanen Zellen, im Gegensatz zu anderen Organen. Dies verunmöglichte rektale HIV-Transmission, sogar in Situationen mit hohem Risiko, wie zum Beispiel in Mäusen mit einer entzündeten Darmschleimhaut. Daher sind humanisierte Mäuse schlecht geeignet um rektale HIV-Transmission zu studieren.

Dieser vermeintliche Nachteil fehlender Immunzellen im Darm erlaubte uns aber einen der wichtigsten HIV-Krankheitsmechanismen zu studieren. Indem wir die humanisierten Mäuse mit HIV infizierten oder die Barrierefunktion des Darmes manipulierten, konnten wir die Situation in chronisch HIV-infizierten Patienten simulieren. Diese Patienten haben auch ein Defizit von CD4+ T-Zellen im Darm. In den humanisierten Mäusen konnten wir nun zeigen, dass aufgrund der HIV-Infektion Darmbakterien in den Körper eindringen und nicht mehr von Makrophagen beseitig werden. Diese Makrophagenfehlfunktion erlaubt eine Aktivierung des Immunsystems, die mit vermehrter HIV-Replikation und einem verschlimmerten Krankheitsverlauf einhergeht. Makrophagenfehlfunktion ist eine wichtige Facette des HIV-bedingten Immunschadens.

Zusammenfassend unterstreichen unsere Resultate die wichtige Rolle des Darmes als HIV-Zielorgon. Beeinflussung von Zellen im Darm oder Zellen, die die systemischen Konsequenzen einer gestörten Darmfunktion regulieren, ist ein vielversprechender therapeutischer Ansatz.

Chapter 1 introduces the role of the intestinal mucosa in HIV infection. **Chapter 2** explores experimental models of rectal HIV transmission. In **chapter 3** the relationships between mucosal dysfunction and HIV pathogenesis are summarized. **Chapter 4** examines the systemic consequences of intestinal barrier breakdown in HIV infection.

1. Introduction

1.1 The intestinal mucosa as a target organ for HIV

HIV infection of intestinal lymphocytes

Already in the beginning of the HIV pandemic, it was suspected that the gut associated lymphoid tissue (GALT) plays a central role for the spreading of HIV. In 1981, physicians in San Francisco noted a clustering of immunodeficiency and opportunistic infections in beforehand healthy young men[1]. Since most of them were homosexual, sexual transmission of an infectious agent via rectal exposure seemed likely[2,3]. Subsequently, a novel retrovirus was identified as the causative agent[4,5] and named human immunodeficiency virus (HIV). Indeed, HIV is often transmitted via mucosal surfaces and unprotected rectal intercourse carries a high transmission risk[6]. Thus, rectal HIV exposure is considered a major risk factor for both homosexual and heterosexual HIV transmission[7]. Even if infection is established by a different route, for example by intravenous HIV exposure, the GALT is a site of extensive viral replication[8]. The GALT provides an optimal environment for HIV to establish and maintain an infection due to its unique cellular composition. HIV mainly infects CD4+ lymphocytes and macrophages[9], and both these cell types are abundantly present in the intestinal mucosa[10].

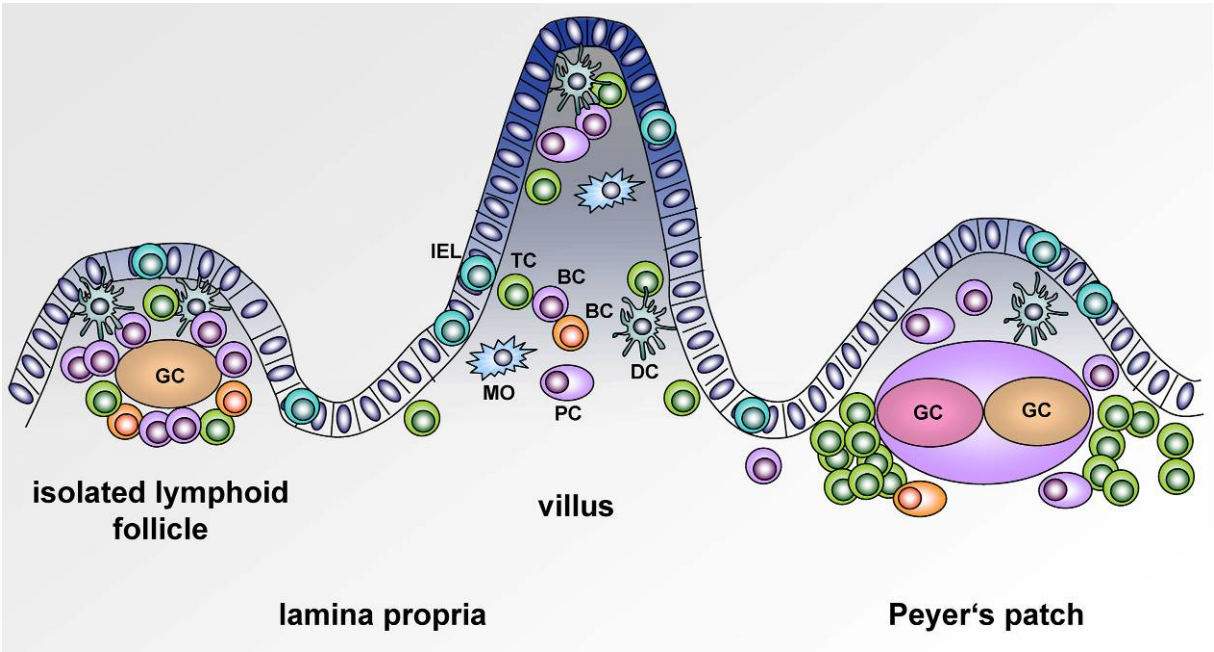


Figure 1: The GALT is densely populated with HIV target cells. The intestinal immune system consists of priming (isolated lymphoid follicles and Peyer's patches) and effector sites (lamina propria). Both contain numerous lymphocytes and macrophages that can be infected by HIV. BC = B cell, DC = dendritic cell, GC = germinal center, IEL = intraepithelial lymphocyte, MO = macrophage, PC = plasma cell

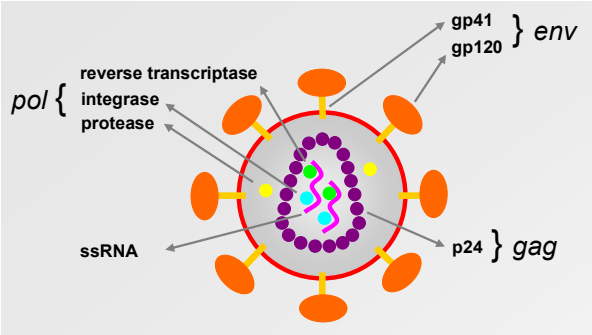


Figure 2: Three main genes influence the structure and function of HIV. The HIV genome consists of ssRNA and encodes for three genes common in all retroviruses and for some accessory genes specific for HIV. *Pol* encodes the viral enzymes, *gag* the structural protein p24, and *env* the surface glycoproteins gp41 and gp120.

A large part of all the body's lymphocytes reside in the gut[11], either in organized lymphoid structures- such as isolated lymphoid follicles or Peyer's patches, or dispersed inbetween intestinal epithelial cells and the underlying lamina propria (Fig.1). Therefore, the GALT is a formidable target organ for HIV. In particular, HIV's cellular tropism[12] explains the widespread infection of GALT lymphocytes[13]. CD4 is the main receptor for glycoprotein (gp) 120[14] (Figure 2), the viral envelope molecule that mediates entry of HIV into host cells. Additionally, the virus needs a co-receptor, either CCR5[15] or CXCR4[16], for stable attachment and fusion of the viral with the cellular membrane (Figure 3). Most of the virions isolated from patients use CCR5[17]; they are so called R5 viruses. Only late in disease course, in about half of all patients, X4 viruses arise[18]. Reasons for this class switch are not clear yet, but in general it is assumed that the virus adapts to the depletion of CD4+CCR5+ target cells and thereby acquires the ability to use CXCR4 as co-receptor[19].

CD4, CCR5 and CXCR4 are expressed on T-helper lymphocytes and most monocytic cells[20]. The exact expression pattern though depends on maturation and activation state of the cells. In the GALT many lymphocytes are in a partially activated, memory state[21] and therefore express CD4 and CCR5[22]. HIV easily infects such cells. In acute infection for instance up to 60% of all GALT CD4+ T cells harbor HIV DNA[23]. Some of these cells produce HIV; some of them are in a resting state and only harbor HIV DNA without active HIV transcription. HIV is a retrovirus[24]; this means that it can establish a latent reservoir by integrating its reverse transcribed genome into the host DNA[25]. In resting cells, the virus can hide undetected by the immune system for a long time[26]. Then, upon activation of the cell viral RNA is transcribed and new virions are produced (Figure 3). Such a productive infection quickly kills lymphocytes[27], but macrophages in particular can produce virus for several days or even weeks[28]. Thus, the GALT with its huge pool of CD4+CCR5+ lymphocytes and macrophages is optimally suited for HIV to spread, multiply, and remain latently integrated. Thus, interactions of HIV with GALT cells have an impact on acute and chronic infection and heavily influence the outcome of HIV disease on an individual and on a population level.

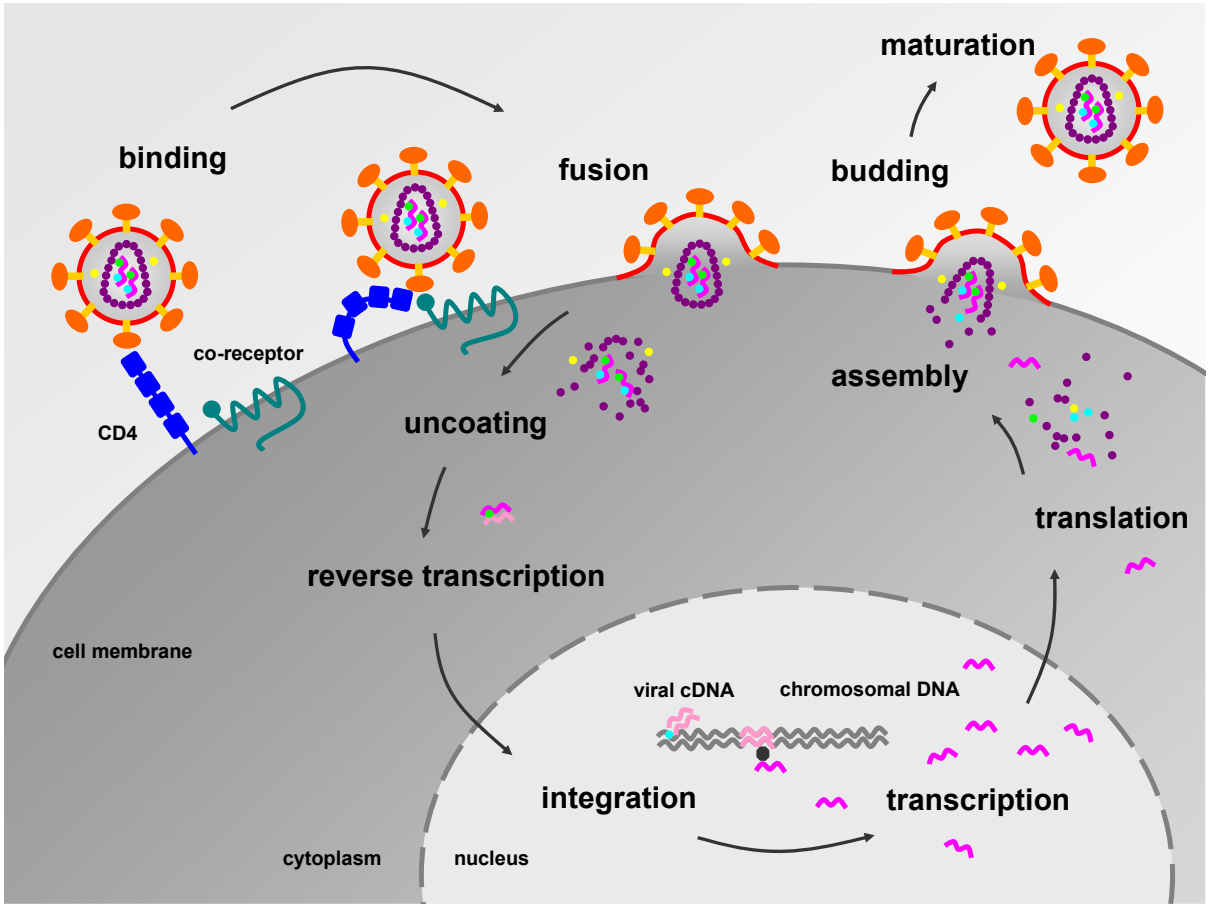


Figure 3: The HIV life cycle depends on binding of the virus to CD4 and a co-receptor. HIV gp120 binds to CD4 and CCR5 or CXCR4. After fusion of the viral membrane with the cell membrane, the viral RNA is uncoated, reverse transcribed and transported to the cell nucleus. The viral genome is integrated into the host DNA and serves as a template for the production of new viral RNA and proteins.

The rectal mucosa as a site of entry for HIV

In most cases, HIV's route of entry is via the mucosa, vaginal, rectal or oral[29]. Transmission by direct contact with blood- i.e. via i.v. drug use, via injuries or medical procedures- is much rarer, although it carries a higher per-contact risk[30,31]. By this direct route, HIV needs not to overcome the mucosal barrier to reach its target cells. The vaginal epithelium with its many layers is quite hard for the virus to overcome. The rectal epithelium, in comparison, is composed of a single layer of columnar epithelial cells and thus, rectal HIV exposure has a relatively high transmission risk compared to other transmission routes[32]. Penetration of the intestinal mucosa can happen by different mechanisms. HIV may enter directly through breaches in the epithelial layer, it may penetrate by transcytosis[33], for example mediated by M cells[34], or dendritic cells, which sample antigen from the intestinal lumen, may take up virus[35]. This last mechanisms is particularly important, since intestinal dendritic cells express

high levels of DC-SIGN, a DC specific C-type lectin, that can bind HIV gp120 and transmit bound virus to T-cells[36]. Certainly, CD4+ T-cells can also be infected directly by free HIV virions, but cell-associated transmission seems to be more efficient *in vitro*[37]. So far, it is not clear which mode of transmission- i.e. cell-free or cell-associated- predominates *in vivo*. In cats, transmission rates were equal in animals inoculated mucosally with cell-free or cell-associated feline immunodeficiency virus[38]. In humans however, the detailed processes leading to rectal HIV transmission are difficult to study. The availability of human intestinal tissue samples is limited, even more so if samples from patients with acute HIV infection are needed.

The risk of acquiring HIV by rectal exposure varies. It increases with the viral load of the transmitter[39], or due to local inflammation[40,41], and traumatic lesions in the exposed. In general though, rectal HIV transmission is relatively ineffective, with an average per-contact risk of about one percent[42,43]. Establishing a new infection via the mucosal route seems to be hard for the virus. This notion is further supported by data about the viral diversity in acutely infected patients. Normally HIV is a very diverse virus with many different viral clones present in a single patient[44]. The reverse transcriptase, that transcribes the viral RNA genome into DNA, is error prone[45] and recombination between different HIV quasispecies occurs frequently[46]. Therefore, HIV virions isolated from a single chronically infected patient are very heterogeneous. In acute infection, however, only one or a few viral clones are present in a single patient, indicating that a "bottleneck" exists[47]. This bottleneck limits transmission and establishment of a new infection to certain viral clones[48]. Viral and host factors influencing this bottleneck remain to be determined and further knowledge is needed to design new preventive strategies

Dysfunction of the intestinal mucosa in chronic HIV infection

HIV infection is characterized by a massive depletion of intestinal CD4+ T-cells[49]. CD4+ T-cells have important functions for regulating mucosal immune defenses. Particularly in the gut, where a multitude of mostly harmless microbes reside, CD4+ T-cells contribute to a fine tuned balance between tolerance and defense[50]. Loss of this protective function might be crucial in determining HIV pathogenesis[51]. HIV's disease course is quite heterogeneous from patient to patient[52] and some of these differences may be due to differences in GALT function and subsequent changes that may influence HIV replication and T-cell loss[51] (Figure 4).

Many patients with chronic HIV infection show signs of enteropathy- diarrhea in the absence of a typical infectious cause[53]. Active viral replication has detrimental effects on intestinal barrier function; both viral components[54] and cytokines[55] produced in response to HIV infection influence the epithelial layer and this results in increased permeability. Translocation of bacterial products from the gut is associated with HIV disease progression in chronically HIV-infected patients[56]. Functional data about bacterial translocation and HIV pathogenesis are rather scarce. Therefore, direct causative links between intestinal barrier dysfunction and HIV pathogenesis are not completely understood yet.

Restoring mucosal barrier function and immune control might prevent HIV pathogenesis. Results from studies in monkeys support this hypothesis. African monkeys infected with simian immunodeficiency virus (SIV) remain healthy despite depletion of intestinal CD4+ T-cells[57]. This species evolved to depend less on CD4+ T-cells for controlling intestinal barrier function[58] and to rapidly limit the pro-inflammatory response to SIV infection[59]. Eliciting such an "African monkey phenotype" in humans might slow down or stop the development of immunodeficiency in HIV-infected patients. But, understanding the exact mechanisms leading to intestinal CD4+ T-cell depletion and subsequent disease progression in HIV-infected humans is a prerequisite to develop such new therapeutic approaches.

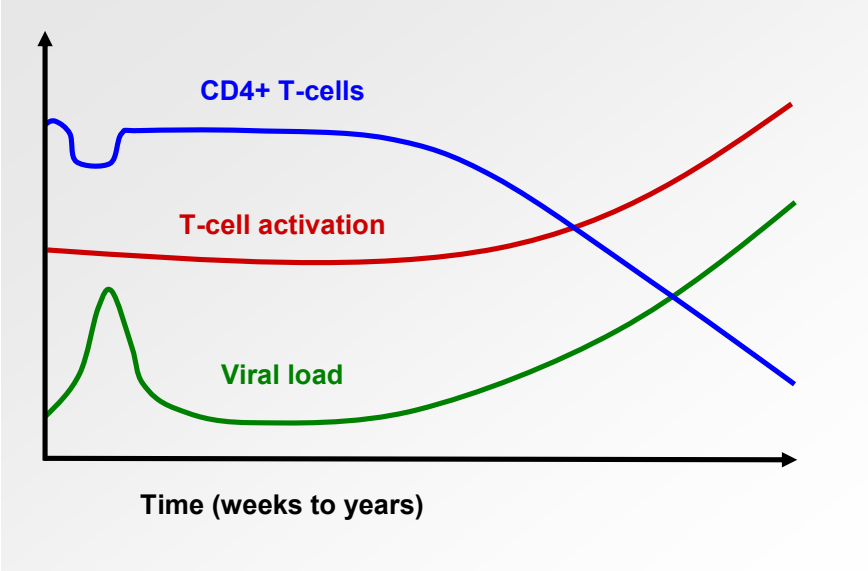


Figure 4. Variances in HIV progression can be explained by activation. After the initial uproar of acute infection, CD4+ T-cell loss and HIV replication gradually progress. Progression rates correlate with T-cell activation levels, which in turn are linked to bacterial translocation. Depending on activation status, a patient's time to developing AIDS varies from weeks to years.

1.2 Aims of the thesis

Establishing a small animal model of rectal HIV transmission

HIV research has long been hampered by the lack of a small animal model that mirrors HIV infection in humans[60] and permits to study questions such as vaginal or rectal transmission. Availability and adaptability of human intestinal tissue explants are limited. Conventional laboratory animals, such as rats or mice, are resistant to HIV infection[61]. Otherwise, SIV infection of rhesus macaques can be used as a model of pathogenic retroviral infection[62]. Although SIV is closely related to HIV, not all results from monkey studies can be directly translated to the human situation. Furthermore, ethical and financial factors foreclose large scale experiments in rhesus macaques. A small animal HIV model would greatly facilitate HIV research *in vivo*.

Transplanting immunodeficient mice with human lymphocytes is a well established strategy to generate HIV permissive mice (Figure 4). Though HIV infection is possible in these mice, depending on the specific model system considerable disadvantages exist. For instance, mice receiving peripheral blood lymphocytes (hu PBL SCID)[63] are easy to generate but human engraftment is limited to a relatively short time, not all cell-lineages are present, and almost all lymphocytes exhibit an activated phenotype due to the xenogenic environment. To achieve longer lasting engraftment fetal organs such as thymus or liver (hu thy/li SCID) can be used for transplantation[64]. But this approach rarely leads to systemic engraftment of human cells, therefore HIV infection remains confined to the transplanted organoid. Combining the transplantation of fetal tissues and hematopoietic stem cells generates bone marrow/liver/thymus (BLT) mice that show systemic human engraftment[65] and even are susceptible to mucosal HIV transmission[66]. But, a model that does not require the transplantation of fetal organs would be much simpler and allow the generation of larger cohorts of animals.

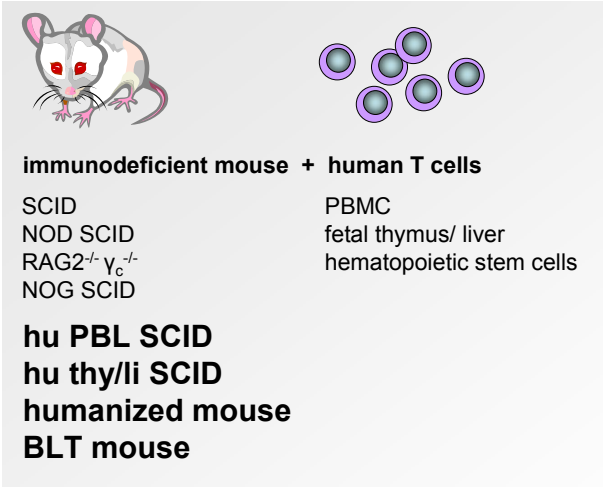


Figure 4: Different HIV mouse models. Several strains of immunodeficient mice are used for transplantation of human T-cells that then can be infected with HIV. Transplanting differentiated lymphocytes from peripheral blood (PBL) generates animals for short term experiments. By using either fetal organs or hematopoietic stem cells, longer lasting human engraftment can be achieved. Properties of each model depend on mouse strain and transplantation protocols.

Therefore, we decided to explore the potential of so called humanized mice as a model for rectal HIV infection. Newborn, immunodeficient mice show good engraftment of human cells after transplantation of hematopoietic stem cells. Human T-, B- and myeloid cells develop de novo and human cells can be found in many organs[67]. Thus, humanized mice support disseminated, long-lasting HIV replication[68]. In the current work, we evaluated human engraftment in the intestinal tract of humanized mice, determined HIV transmission rates after rectal inoculation, and assessed the impact intestinal inflammation on HIV transmission (**Chapter 2: Rectal HIV transmission in humanized mice**).

Investigating the pathogenic potential of bacterial translocation in HIV infection

The natural course of HIV disease is a progressive destruction of the immune system eventually leading to the acquired immunodeficiency syndrome (AIDS)[69]. The hallmark of this immunodeficiency is the progressive loss of CD4 T-cells[27]. The mechanisms leading to CD4 T-cells loss in chronic HIV infection remain poorly understood. During the chronic phase, only small fraction of all CD4 T-cells actively produce HIV[70], indicating that only a minority of cells may be killed by direct viral cytopathicity. This led to the notion that most of the cells that die are in fact not infected[71]. Currently two mechanisms are held responsible for T cell death: one proposes chronic immune activation and activation induced cell death (AICD) as the driving force behind HIV pathogenesis; the other implies that released viral proteins such as gp120, Tat, Vpu or Nef induce killing of bystander cells[27].

There is growing evidence in support of the chronic immune activation theory. Up-regulation of the cell surface activation marker CD38 on CD8 T cells is the best prognostic factor for disease progression[72]. Furthermore, in monkey studies a good correlation was found between

immune activation and disease development[73]. Briefly, African monkeys infected with simian immunodeficiency virus (SIV) do not show immune activation and do not progress to AIDS. In contrast, SIV infection of Asian monkeys leads to immune activation and disease progression. However, the causes of immune activation are poorly defined.

A potential mechanism causing immune activation is invasion of microbial products from the gut. Circulating lipopolysaccharide (LPS), a marker of microbial translocation from the gastrointestinal tract, is increased in chronically HIV infected individuals and correlates with immune activation levels[56]. However, the question remains if this microbial translocation is the cause or rather an effect of the immune disturbance seen in HIV infection (reviewed in **Chapter 3: Pathogenesis mechanisms of bacterial translocation in HIV infection**).

So far most studies investigating bacterial translocation in HIV infection have only shown correlative data from infected humans or monkeys. Consistent findings have been that in chronic infection plasma LPS is increased and that these values correlate with immune activation, which in turn is the best marker to predict disesease progression. Up to now no animal model was available to study the causative relations between bacterial translocation and HIV pathogenesis. Our unique model of HIV infected humanized mice allows to investigate these connections in a targeted and well controlled manner. In the current work, we elucidated the relationships between intestinal permeability, bacterial translocation, clearance of translocated bacterial products, and HIV pathogenesis (**Chapter 4: Bacterial translocation and immune activation in HIV infected humanized mice**).

2. Rectal HIV transmission in humanized mice

Published as Hofer et al., J Virol 2008; 82(24):12145-53.

$RAG2^{-/-}\gamma_c^{-/-}$ mice transplanted with human cord blood CD34+ cells show low intestinal engraftment and are resistant to rectal HIV transmission

Ursula Hofer[1]*, Stefan Baenziger[1], Mathias Heikenwalder[2], Erika Schlaepfer[1], Nadine Gehre[1,3], Stephan Regenass[4], Thomas Brunner[5], Roberto F Speck[1]*

[1]Division of Infectious Diseases and Hospital Epidemiology and [2]Institute of Neuropathology, University Hospital Zurich, [3]Experimental Infectious Diseases and Cancer Research, Division of Infectious Diseases and Hospital Epidemiology, University Children's Hospital Zurich, [4] Division of Clinical Immunology, University Hospital Zurich, Zurich, Switzerland, [5]Institute of Pathology, Division of Immunopathology, University of Bern, Bern, Switzerland

Rectal transmission is one of the main routes of infection by human immunodeficiency virus type-1 (HIV). To efficiently study transmission mechanisms and prevention strategies, a small animal model permissive to rectal HIV transmission is mandatory. We tested the susceptibility of $RAG2^{-/-}\gamma c^{-/-}$ mice transplanted with human cord blood hematopoietic stem cells to rectal HIV infection. We rectally exposed these humanized mice to cell-free and cell-associated HIV. All mice remained HIV negative as assessed by plasma viral load. The same mice infected intraperitoneally showed high levels of HIV replication. In the gut-associated lymphatic tissue, we found disproportionately smaller numbers of human cells than in other lymphoid organs. This finding may explain the observed resistance to rectal HIV transmission. To increase the numbers of local HIV target cells and the likelihood of HIV transmission, we treated mice with different pro-inflammatory stimuli: local application of interleukin 1β, addition of seminal plasma to the inoculum or colitis induction with dextran sodium sulphate. These procedures attracted some human leukocytes, but the transmission rate was still very low. The humanized mice showed low human engraftment in the intestinal tract, seem to be resistant to rectal HIV transmission, and thus are an unsuitable model for this application.

Introduction

Millions of people worldwide are infected with HIV. Despite improved treatment strategies and efforts in prevention, no end of the pandemic is in view. HIV transmission occurs mostly by sexual intercourse via mucosal routes[74]; rectal intercourse carries an especially high risk[6] and thus is considered one of the major risk factors for both homosexual and heterosexual HIV transmission[7]. For the rational design of prevention methods such as microbicides, we need detailed knowledge of HIV transmission and in particular the early steps of HIV infection.

HIV research has long been hampered by the lack of a small animal model that recapitulates HIV infection in humans and permits to study vaginal or rectal transmission. Studies with larger animals can provide important clues to understand transmission and improve the design of trials in humans. Non-human primates and less frequently cats have been used[62,75], but have significant problems, such as low availability, high costs and species differences. HIV is a human-specific virus, and non-human primates and felines are resistant to HIV. Thus, either simian, simian-human chimeric or feline immunodeficiency viruses have to be used as an approximation for HIV.

Immunodeficient mice transplanted with human cells as an animal model for HIV disease have numerous advantages and some shortfalls. Cohort size can be increased significantly, providing more robust statistical validity to experiments. Mice are less costly and labor intensive than larger animals, and notably, they are susceptible to HIV. Severe combined immunodeficient (SCID) mice transplanted with either human fetal thymus/liver (Thy/Li) or human peripheral blood leukocytes (PBL)[76] are well-established models. The rate of mucosal HIV transmission in hu-PBL-SCID mice is low and variable[77] and renders this mouse model unsuitable for preclinical studies of microbicides. Significantly, the SCID-hu Thy/Li mouse has never been evaluated for mucosal transmission since human cells are mostly found in the transplanted organ and peripheral engraftment is low[78].

Recently, we and others reported humanized mice as a promising new model for HIV research[66,68,79-83]. In these models, transplantation of human hematopoietic $CD34^+$ cells into immunodeficient mice leads to the development of all human lymphoid lineages and the repopulation of lymphoid organs with human cells. These models differ according to the mouse strains used (i.e., NOD/SCID, NOG/SCID or $RAG2^{-/-}c_\gamma^{-/-}$ mice) and the origins of human hematopoietic cells transplanted (i.e., $CD34^+$ from fetal liver or cord blood). Previously, we showed sustained and disseminated HIV replication in $RAG2^{-/-}c_\gamma^{-/-}$ mice

transplanted with cord blood $CD34^+$ cells after intraperitoneal injection of HIV[68]. So far, only two studies investigating HIV infection in humanized mice showed successful HIV infection by the rectal route[66,84]. In these studies, human transplants were obtained from fetal tissue. Aside from any ethical considerations, fetal tissue is not as easily and widely available as cord blood. Furthermore, one study used bone marrow/liver/thymus (BLT) mice, which receive a thymic organ transplant before irradiation and reconstitution with fetal liver derived hematopoietic stem cells.

In the current study, we examined mucosal HIV transmission in $RAG2^{-/-}c_\gamma^{-/-}$ mice transplanted with cord blood-derived $CD34^+$ cells. We characterized the engraftment of human cells into the gastrointestinal tract of these humanized mice and determined their susceptibility to rectal HIV transmission. We challenged mice with cell-free and cell-associated HIV since their relative contributions are not known. Overall, rectal transmission rates were low in all groups of humanized mice, independently of pre-infection treatment and inoculation protocols.

Material and methods

Generation of humanized mice

Mice were reconstituted as described[67,68]. Briefly, newborn RAG2$^{-/-}$c$_\gamma^{-/-}$ mice were irradiated with 2x2 Gy. CD34$^+$ cells were isolated from cord blood with immunomagnetic beads (Milteny Biotec), and 150,000 to 400,000 cells (246,000 ± 60,000) were transplanted into each mouse. Fetal liver derived CD34$^+$ cells were a kind gift from R. Akkina (Colorado State University). Eight to 12 weeks after transplantation, the levels of blood engraftment were determined by flow cytometry of peripheral blood mononuclear cells (PBMCs) stained for the human panleukocyte marker CD45 in all mice (mean human cells/live cells 11.56 ± 10.9%). Liver, bone marrow, and spleen engraftment levels were also analyzed in 14 mice. All experiments were approved and conducted according to local guidelines and laws.

Tissue characterization

Formalin-fixed tissue sections from liver and large intestines were stained with hematoxylin and eosin (H&E) or for markers for human CD45 (clones PD7/26 and 2B11, Dako), CD4 (clone 1F6, Novacostra) and CD68 (clone GG-M1, Dako). To control for non-specific staining, tissue from untransplanted mice was stained as well. Human CD45$^+$ cells were counted at a magnification of 200x at 10 randomly chosen locations per organ and mouse. In interleukin (IL)-1β-treated mice, cell numbers were evaluated separately for the 1.5 cm most proximal to the anus.

Lymphocytes from the small and large intestines were isolated (gut-associated lymphatic tissue or GALT)[85] sequentially from the intraepithelial and lamina propria compartments of three mice with a mean blood engraftment of 13.7%. Briefly, intestines were opened longitudinally, cut in pieces and washed five times with cold PBS, 2% FCS. Then the epithelial layer was dissociated by stirring tissue pieces for 30 minutes in PBS, 2% FCS, 2 mM EDTA and 2 mM dithiothreitol at 37° C. Supernatants were collected and filtered through 70- and 40-µm cell strainers. Cells were washed twice and kept on ice until analysis by flow cytometry. The remaining tissue was incubated for 15 minutes in cold RPMI, 10% FCS, then stirred at 37° C in RPMI, 5% FCS, 100 U/ml collagenase IV and 10 U/ml DNAse I (both from Sigma) to extract lamina propria lymphocytes. After 30 and 60 minutes of digestion, supernatants were collected, and fresh digestion medium was added to the tissue pieces after the first collection step. Supernatants were treated as mentioned above.

Pro-inflammatory treatment with interleukin 1β

Mice were anaesthetized with Ketamine and Rompun (50 and 8 mg/kg, respectively), and 250 units of recombinant human interleukin (IL)-1β (Invitrogen) in 25 µl of PBS were applied intrarectally with a pipette 24 hours before infection. The mice were suspended head down for 1 minute.

Colitis induction

Colitis was induced by cyclic treatment with 1% (w/v) dextran sodium sulfate (DSS) (molecular weight 40,000, MP Biomedicals) added to the drinking water for 4 days, followed by 7 days of normal drinking water[86,87]. At the end of the third cycle, colitis was verified in formalin-fixed tissue sections, or the mice were used for HIV challenges. H&E sections of DSS-treated and untreated mice were scored for epithelial changes and inflammatory infiltration as described[88].

HIV infections

For cell-associated HIV infections, the CCR5-tropic HIV strain YU-2 was used, for cell-free HIV infections either YU-2, JR-CSF (CCR5-tropic), NL4-3 (CXCR-4 tropic) or 89.6 (dual tropic)[68] were used. Cell-associated HIV was prepared by infecting $CD8^+$ T cell-depleted PBMCs pooled from three donors. PBMC first were stimulated with 2 µg/ml phytohemagglutinin (PHA) for 3 days, then spinoculated with YU-2 at an MOI of 0.1 for 2 hours, at 1200g and 25° C. After the washing, the cells were returned to culture without further addition of PHA. Four days after spinoculation, infection rates were measured by intracellular p24 staining (6.5–12.5% positive), and viability was controlled by Trypan blue staining (>90%). Cells were washed twice and suspended either in PBS or seminal plasma. Cell-free virus stock was used undiluted or mixed 1:1 with seminal plasma. Mice received rectally cell-free stock virus at a dose of $2x10^5$ (YU-2), $5x10^3$ (JR-CSF), $1x10^4$ (89.6) or $2x10^5$ (NL4-3) $TCID_{50}$ or $2x10^6$ infected PBMCs. Anesthesia and inoculation were performed as described above. 20 µl of the respective inoculum were applied per mouse, except for mixtures of cell-free HIV and seminal plasma, which were given in volumes of 40 µl. Mice infected intraperitoneally received 200 µl of cell-free virus, corresponding to $2x10^6$ $TCID_{50}$. Plasma viral loads were measured by RT-PCR (Amplicor, Roche) at 4 to 6 weeks and again at 8–12 weeks post infection (p.i.). Detection limit was 800 copies/ml.

Tracking of rectally instilled labeled cells

PBMC were stimulated with PHA, mock spinoculated and cultured as above. Cells were labeled with carboxy fluorescein succinimidyl ester (CFSE), washed and mixed in 20 µl of either PBS or seminal plasma and applied rectally at $2x10^6$ cells per mouse. After 2, 4, and 6 hours, the mice were euthanized. Intestinal tissue was removed and snap-frozen. Cryosections were ethanol fixed and counterstained with DAPI.

Statistical analysis

GraphPad Prism 5.01 was used for statistical analysis. Data were analyzed by two-tailed t-tests, all unpaired except for naive mice, in which cell numbers from liver and rectum from the same mouse were paired.

Results

Low engraftment efficiency of human cells in the intestinal tract of humanized mice

GALT contributes substantially to the lymphoid system overall, and thus, its detailed characterization is mandatory for estimating its contribution in immunological questions. In humans, GALT contains abundant numbers of $CD4^+$ T cells, the main target cells of HIV. However, little is known about GALT in humanized mice.

We examined the engraftment of human cells into the gut of $RAG2^{-/-}c_\gamma^{-/-}$ mice transplanted with $CD34^+$ cells from human cord blood. Tissue sections of the rectum and the liver of 14 humanized mice were fixed with formalin and stained for the human panleukocyte marker CD45. Isolated cells from the liver, bone marrow, blood and spleen were analyzed for $CD45^+$ cells by flow cytometry. From three additional mice, we isolated GALT cells: intraepithelial lymphocytes and lamina propria lymphocytes from the small (SI IEL and SI LPL) and large intestines (LI IEL and LI LPL). We included three wild-type mice as positive controls for the isolation procedure of the GALT.

Human cell numbers in tissue sections of rectum were significantly lower than in those from liver (Fig. 1A and B). The same pattern was seen in flow-cytometric analysis: engraftment levels in the intestines ranged between 0.001 and 0.1%, whereas in other organs examined, they were up to 800 times higher (Fig. 1D). The cellular composition and the protocols to isolate cells differ substantially, according to tissue. Thus, comparisons between relative cell numbers in organs are difficult to interpret. However, GALT lymphocytes from wild-type mice exhibit large numbers of $CD4^+$ cells in the LI LPL cell fractions (Fig. 1C). Isolation of $CD4^+$ cells from wild-type mice shows that the lack of human cells in the GALT from humanized mice is not due to technical problems.

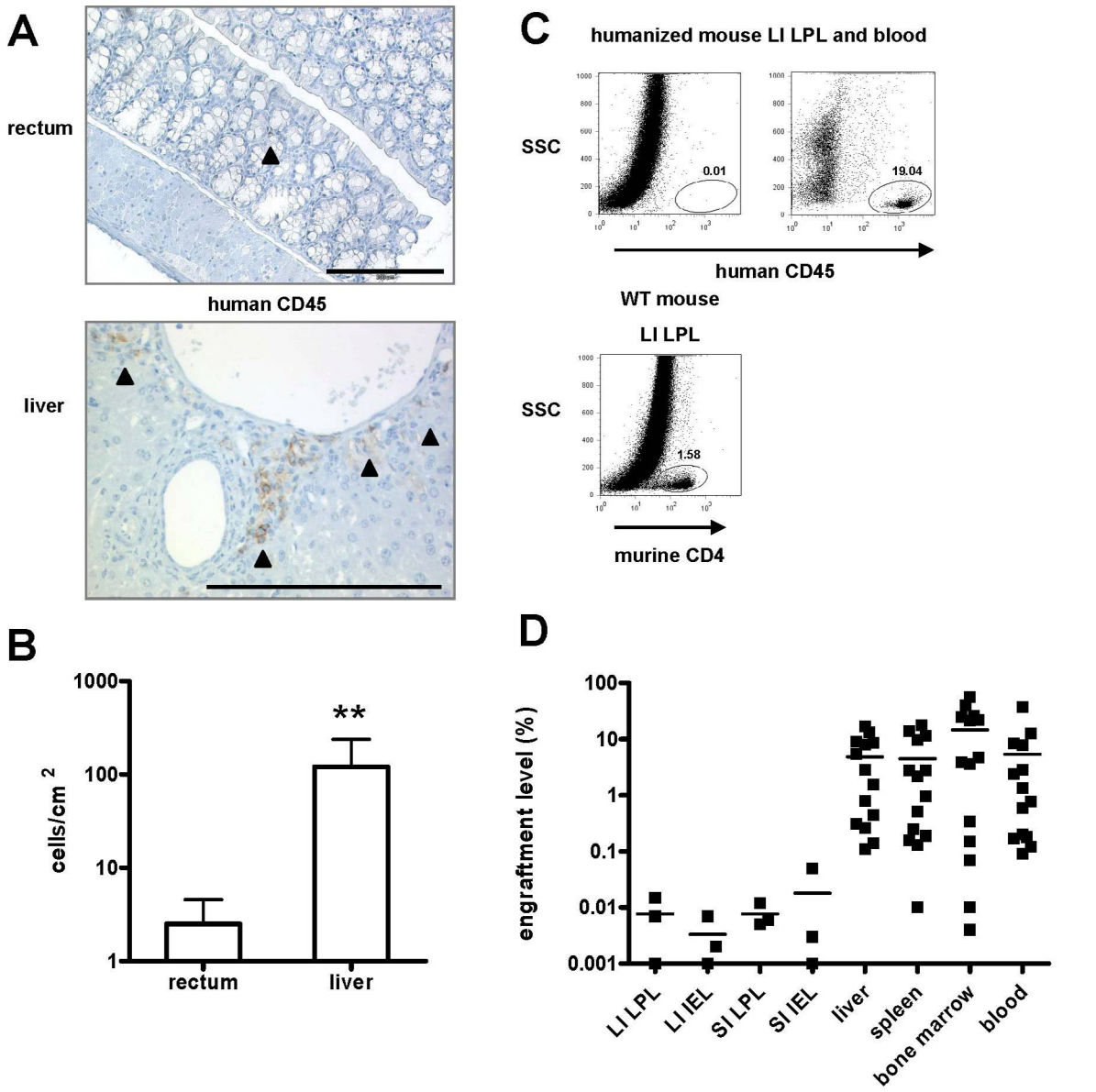


Figure 1: Engraftment levels were disproportionately lower in the gut of humanized mice than in other tissues. Mice were analyzed for engraftment with human leukocytes by measuring cells positive for the human panleukocyte marker CD45. Tissue sections of rectum and liver of 14 mice were stained for CD45 (A, arrows indicate positive cells), and cells were counted per square centimeter (B, mean, SD, p=0.0094). From three mice, lymphocytes from the intraepithelial space (IEL) and the lamina propria (LPL) from the large and small intestine (LI and SI) were isolated. Cells from the liver, spleen, bone marrow, and blood were isolated from 14 mice and analyzed by flow cytometry for proportions of human CD45-positive cells. Despite high engraftment levels in the blood, almost no human cells were detectable in the lamina propria of the large intestines, where the majority of $CD4^+$ cells were found in wild-type mice (C). Overall, intestinal engraftment was low, whereas in other organs, percentages of human cells were high (D). Bars represent 200µm.

Naive humanized mice are resistant to rectal HIV transmission

In body fluids, HIV occurs as free virions (cell-free) or inside of infected cells (cell-associated virus). The extent of cell-free or cell-associated HIV in rectal transmission is unknown. Infected cells could act as "Trojan horses" to release large numbers of virions upon invasion of mucosal tissue.

We exposed five naive (untreated) humanized mice to cell-free HIV YU-2 at a dose of $2x10^5$ $TCID_{50}$ per mouse and nine mice to cell-associated HIV YU-2 (i.e., $2x10^6$ HIV-infected PBMC, range of HIV-positive PBMC as verified by intracellular p24 staining: 6.5–12.5%) per mouse. After 4 weeks, all mice were negative for plasma viral load by PCR. We then injected four of the same mice with cell-free HIV YU-2 intraperitoneally. These four mice then showed high viral loads, while the remaining rectally exposed mice still were negative 8 weeks after inoculation (Fig. 2B). We included three mice with no detectable human engraftment in the blood to verify whether repopulation with rectally instilled HIV-infected cells would result in HIV infection. These also tested negative for HIV.

It is possible, that the origin of human stem cells used to generate humanized mice could influence intestinal engraftment efficiency and subsequently rectal HIV infection rates. Therefore we reconstituted nine newborn mice of the same litter either with cord blood derived or fetal liver derived $CD34^+$ cells and 12 weeks later exposed them rectally to cell-free HIV YU-2 as described above. One of the five mice which received cord blood cells and none of the four mice reconstituted with liver derived cells tested HIV positive one month after challenge (Fig.2B).

The efficiency of rectal transmission varies between different HIV strains. To exclude that the low rectal transmission rates we observed, were a specific feature of YU-2, we tested three other viral strains: JR-CSF, 89.6 and NL4-3. Three groups of six mice received cell-free HIV rectally at a dose of $5x10^3$ (JR-CSF), $1x10^4$ (89.6) or $2x10^5$ (NL4-3) $TCID_{50}$ per mouse. Of the 18 mice challenged none showed a detectable plasma viral load 4 and 8 weeks after challenge.

We also prepared PBMC for mock infections. These cells were labeled with CFSE before rectal instillation to track their location subsequent to rectal instillation (Fig. 2A). Only a few single cells had invaded the mucosa after 6 hours; most of the inoculum probably was excreted (Fig. 2C).

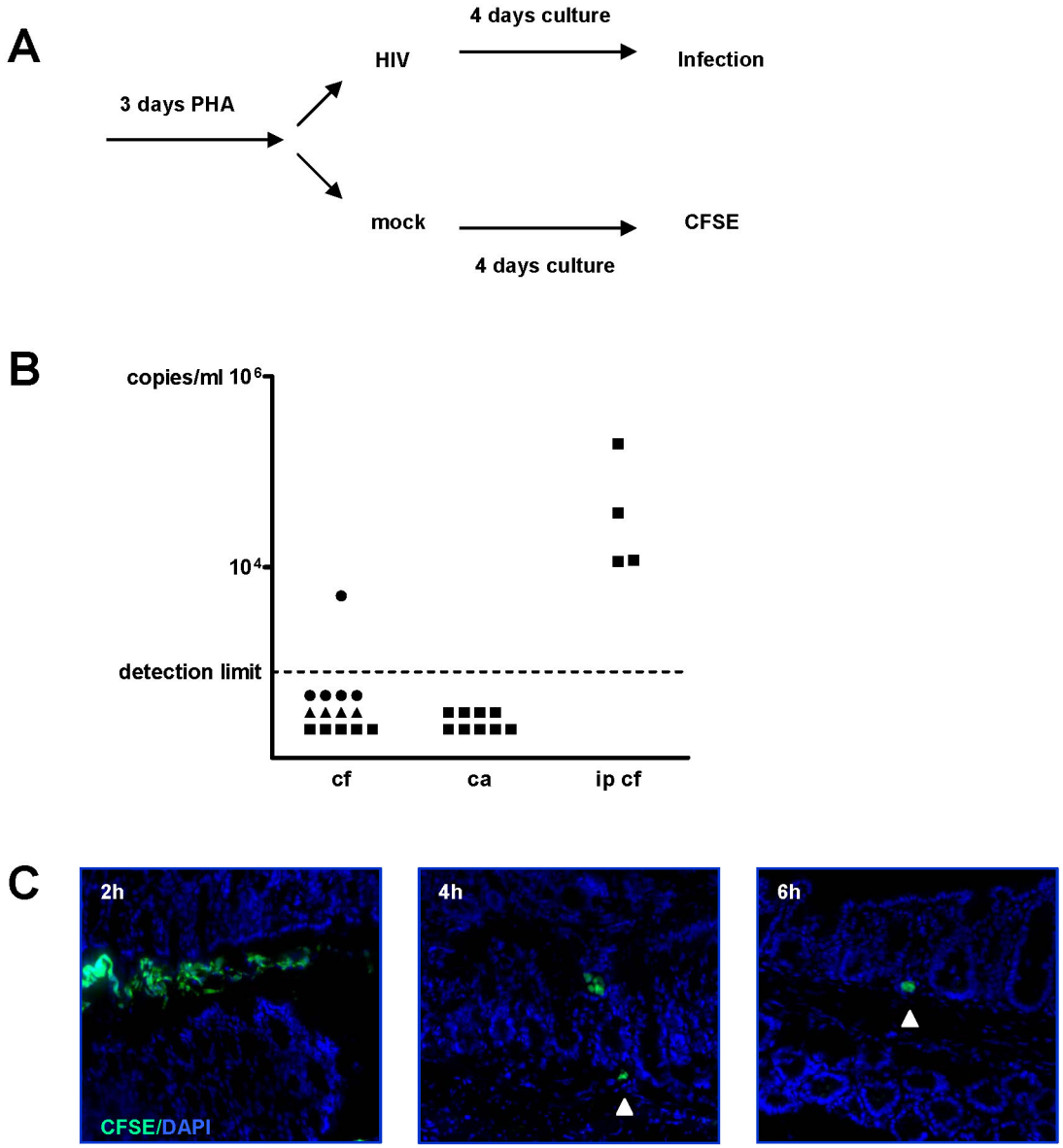


Figure 2: Rectal HIV transmission in naive mice was rare. Naive (untreated) humanized mice were exposed rectally to cell-free (cf) or cell-associated (ca) HIV, and plasma viral load was measured 4 to 8 weeks later (Amplicor Roche). Cell-associated HIV was prepared following a three-step protocol: $CD8^+$-depleted PBMCs from three donors were pooled and PHA stimulated, then spinoculated and cultured for 4 more days (A). Nine mice were inoculated rectally with infected PBMC; five mice were inoculated with cell-free HIV. Plasma viral load was negative in all mice, four of the same mice were then inoculated by intraperitoneal injection, and all became HIV positive (B, squares). Additional mice were reconstituted in parallel either with fetal-liver derived (B, triangles, n=4) or with cord blood-derived $CD34^+$ cells (B, circles, n=5) and challenged rectally with cell-free HIV. One of the mice receiving cord blood cells tested HIV positive. A cell migration assay was performed with mock spinoculated cells. CFSE-labeled cells were applied rectally to three mice. In cryosections, a few single invading leukocytes (arrows) were detected (C).

While infection rates after i.p. injection of HIV were high, we rarely detected HIV transmission after rectal exposure, either to cell-free or cell-associated HIV. Our finding that instilled cells rarely cross the rectal mucosa can explain the resistance of humanized mice to cell-associated HIV.

Rectal application of IL-1β leads to low levels of infiltration with human cells

IL-1β is a potent pro-inflammatory cytokine and mediates leukocyte chemotaxis. We speculated that local pre-treatment with IL-1β would attract human lymphoid cells to the rectal mucosa and increase HIV transmission. In some experiments, we added human seminal plasma to the inoculums. Seminal plasma may have chemotactic potential[89] and may enhance attachment of HIV to target cells[90]. However, semen has many pro- and anti-viral factors[91], and its exact role in HIV transmission is still unknown.

We treated three humanized mice rectally with 250 units of IL-1β. After 24 hours, we detected inflammatory changes, such as vessel dilatation and cell infiltration, at the site of application (Fig. 3A). Most of the infiltrating cells were murine, but significantly more human cells were found than in untreated mice (Fig. 3A and B). We detected both human CD4- and CD68-positive cells in the rectal mucosa at the site of IL-1β application (data not shown). This observation confirms that IL-1β attracted some human CD4 cells and macrophages, potential HIV targets, to the rectal mucosa of humanized mice.

We also pre-treated mice with IL-1β and challenged them 24 hours later with cell-associated HIV or cell-free HIV, with or without seminal plasma, at the same doses as the naive mice. Transmission rates were still low (Fig. 3C). Of 12 mice inoculated with cell-free or cell-associated HIV mixed with seminal protein, none developed viremia, while one of 5 mice inoculated with cell-free virus developed viremia.

Additionally, we tracked CFSE-labeled cells after rectal application with the IL-1β pre-treatment scheme and addition of seminal plasma to the inoculum. The results were similar to those described for naive mice: only a few single cells crossed the rectal epithelial layer and penetrated into the mucosa (data not shown).

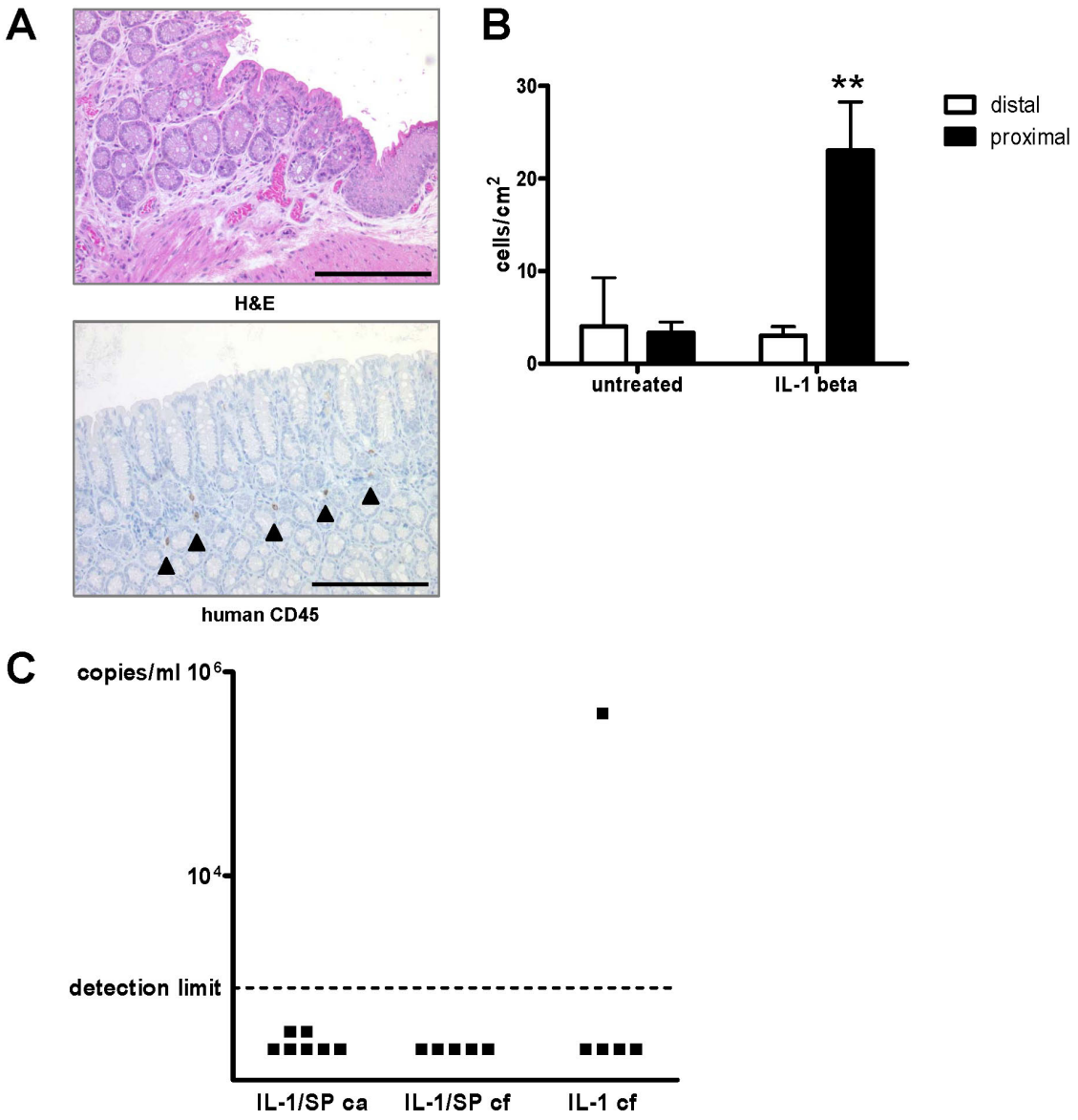


Figure 3: Rectal HIV transmission in mice treated with local pro-inflammatory stimuli. We treated three humanized mice rectally with 250 IU IL-1β and checked for inflammation and infiltration with human $CD45^+$ cells 24 hours later (A, arrows indicate positive cells). Rectal mucosa proximal to application-site showed an increase in human cells, whereas cell numbers distal to application-site were as low as in untreated mice (B, mean, SD, p=0.0033). Mice treated the same way were then exposed to cell-associated (ca) or cell-free (cf) HIV. In some experiments, seminal plasma (SP) was added to the inoculum. Plasma viral load was measured. One mouse showed viral replication 4 weeks after HIV challenge (C), and the same results were seen 8 weeks post infection (not shown). Bars represent 200µm.

Humanized mice are highly susceptible to DSS-induced colitis

Concomitant sexually transmitted diseases are important risk factors for HIV transmission. In particular, mucosal inflammation increases susceptibility to infection by disrupting the epithelial lining and changing numbers and phenotype of local target cells[41]. We tried to reproduce this situation in our humanized mice by inducing a chronic colitis with DSS[87].

So far, DSS colitis has not been studied in humanized mice. Prior studies established that $Rag2^{-/-}$ mice are susceptible to acute[92] and chronic colitis[93] in the DSS model. In a first experiment, humanized mice treated with 3% DSS in normal drinking water for 4 days lost ≥20% of starting weight and showed severe clinical symptoms. Therefore, we treated the humanized mice with three cycles of 1% DSS interrupted by 7 days of normal drinking water and then monitored weight and state of health daily (Fig. 4A). Mice tolerated this reduced dose well and suffered only from minimal weight loss. One week after the last DSS administration, we analyzed the large intestines of three mice histologically: tissue sections showed inflammatory changes with alteration of the epithelial layer and infiltration of leukocytes, partly of human origin (Fig. 4B–D). Notably, human cell numbers in the mucosa seen by staining for human CD45 were significantly higher in DSS treated animals than in animals that received normal drinking water.

To assess the impact of rectal inflammation on HIV transmission rate, we challenged mice with cell-associated HIV 7 days after the last DSS administration. Unexpectedly, 24 hours after inoculation, all mice were severely affected, and some did not recover. Mortality was 50% in the first week after infection. The remaining animals recovered completely, and HIV RNA levels were measured 4 and 8 weeks after HIV inoculation. No infection was detectable in all three mice that completed the trial. We conclude that, despite mucosal inflammation, transmission of cell-associated HIV is improbable in humanized mice.

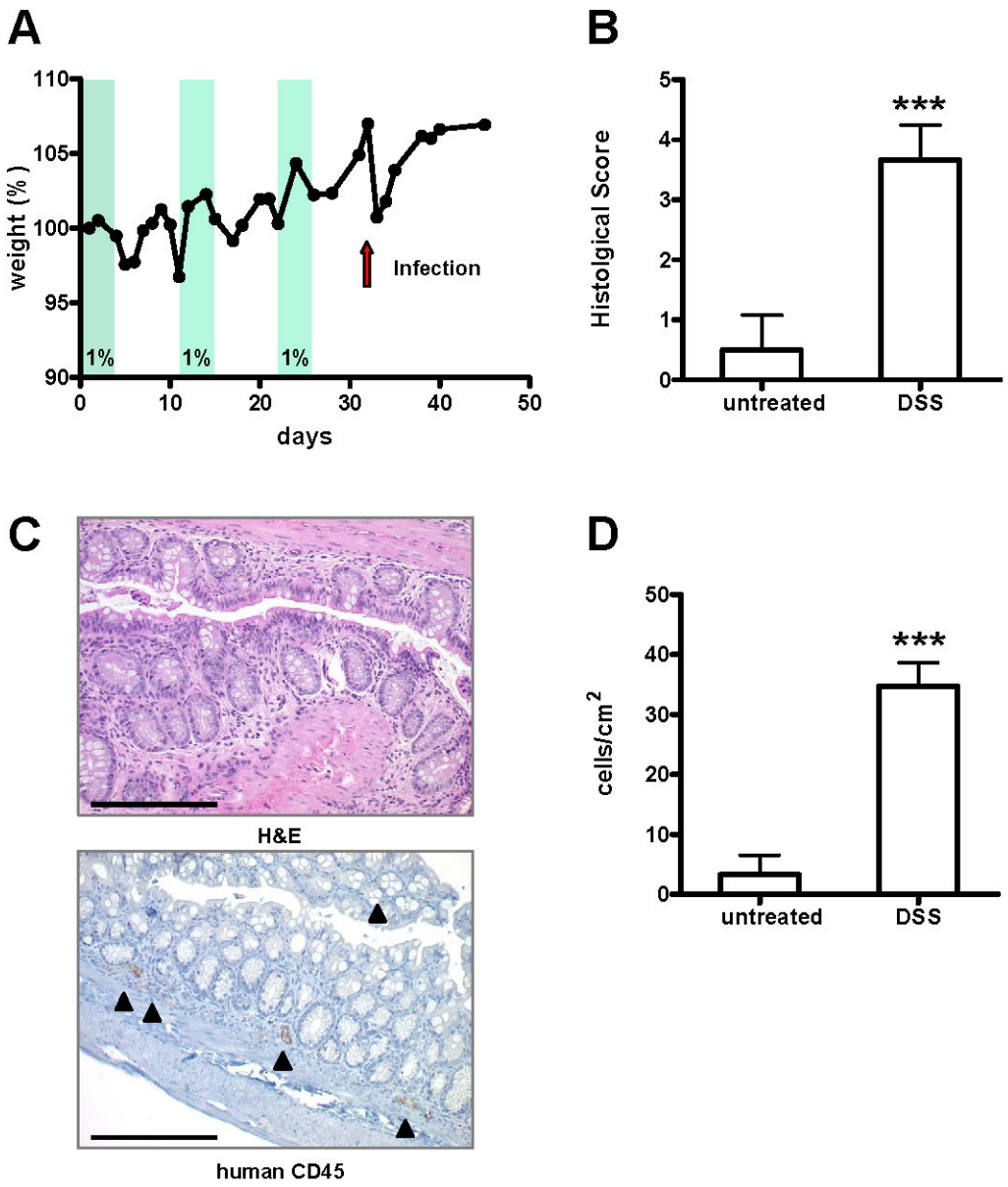


Figure 4: DSS induces colitis in humanized mice. Twelve humanized mice received three cycles of 1% DSS in normal drinking water. This treatment induced moderate weight changes (A) due to colitis induction. Colitis was confirmed by histological scoring of tissue sections of three mice (B, p=0.0008). DSS treatment induced cellular infiltration consisting mostly of murine cells since only a fraction of cells was positive for human CD45 (C, positive cells indicated by black arrows). However, in comparison to untreated mice, human cell numbers were increased after DSS treatment (D, p=0.0005). DSS-treated mice were then inoculated with HIV (red arrow in A). Bars represent 200µm.

Discussion

Humanized mice are a promising model for large-scale studies of HIV transmission, and their characterization is critical to future studies. Our study describes human engraftment in GALT of cord blood $CD34^+$ cell-transplanted $RAG2^{-/-}c_\gamma^{-/-}$ mice. We show that, in this particular model, human cell numbers in the GALT are lower than in other lymphatic organs and increase only moderately after pro-inflammatory treatment. Furthermore, we examined the susceptibility of these humanized mice to rectal HIV transmission and found a very small rate of rectal HIV transmission. Only 2 of 56 challenged mice became HIV positive. Thus, this model may have limited value for the study of rectal HIV transmission.

Two other studies showed successful rectal infections of humanized mice[66,84] with transmission-rates of 6/7 and 11/14, respectively. Sun et al.[66] used NOD/SCID mice transplanted with fetal liver and thymus and later reconstituted with liver-derived $CD34^+$ cells (BLT mice). Berges et al.[84] transplanted $RAG2^{-/-}c_\gamma^{-/-}$ mice with $CD34^+$ cells from fetal liver. Thus, a major difference is that we used cord blood–derived cells. So far it is not clear which source of hematopoietic stem cells is optimal for the generation of humanized mice. *In vitro*, the colony-forming efficiency of $CD34^+$ cells derived from fetal liver is higher than that of cord blood $CD34^+$ cells[94]. However, *in vivo* cord blood–derived hematopoietic stem cells produced four times more mature human leukocytes than liver-derived cells, when the same number of stem cells was transplanted into adult NOD/SCID mice[95]. When telomere length is used as a measure of replicative capacity of hematopoietic stem cells from fetal liver and cord blood, the differences are minimal[96], indicating that both are suitable for generating humanized mice. However, it is not known to what extent the origin of lymphoid cells may be critical for repopulation of the intestines with human cells. Different expression of cell adhesion molecules[97] could influence homing behavior and subsequent repopulation of lymphoid organs. That, in turn, may explain the lower infection rates we observed in our model using cord blood–derived cells for transplantation. But when we reconstituted mice in parallel either with cord blood-derived or with fetal liver-derived cells, rectal HIV transmission was not more efficient in the mice which had received liver-derived cells.

Apart from the different origin of $CD34^+$ cells, Berges et al. and we used similar models based on mice with the same genetic background. However, Berges et al. reported higher engraftment levels than the ones we detected. They found blood engraftment levels of almost 90%, whereas the mice used in our study had a mean engraftment of about 11%. But these values cannot be directly compared. Berges et al. measured percentage of human cells in

blood lymphocytes; we measured percentage of human cells in all blood leukocytes, which include murine cells as well. Since RAG2$^{-/-}$c$_\gamma^{-/-}$ mice have no B, T and NK cells of their own, obviously all lymphocytes detected in humanized mice should be of human origin. In our cohort of mice transplanted with liver-derived cells, we saw no differences in engraftment levels compared to engraftment levels in littermates transplanted with cord blood-derived cells. This indicates that the origin of the stem cells probably has no major impact, at least in our hands, and that other factors are influencing HIV transmission rates in humanized mice.

The intestinal microflora plays important roles in GALT development: intestinal bacteria influence the migration pattern of lymphocytes into mucosal sites; in germ-free animals, intestinal lymphocyte numbers are drastically decreased[98]. Certainly, the microflora in the gut of mice from different genetic backgrounds and from different animal facilities varies and could influence human engraftment in GALT and subsequently HIV transmission susceptibility. Besides of potential differences in gut microflora, especially in BLT mice, gastrointestinal engraftment appears to be significantly better than the GALT engraftment we obtained in RAG2$^{-/-}$cγ$^{-/-}$ mice. BLT mice have the advantage of a human thymic tissue transplant, which could facilitate T cell development.

In our humanized mouse model, T-lymphocyte development occurs in the anlage of the murine thymus, which is almost completely repopulated with human cells. Nonetheless, the stroma of this thymus is of murine origin and may have unknown disadvantageous effects on overall lymphoid development. Furthermore, to generate BLT mice, the NOD/SCID mouse strain was used. In contrast to RAG2$^{-/-}$cγ$^{-/-}$ mice, NOD/SCID mice do not have mutations or loss of γ_c, the common gamma chain in receptors of multiple cytokines, including IL-7. Signaling through the IL-7 receptor is essential for formation of the Peyer's patches anlagen[99], and humanized mice lacking this important feature may have more difficulties in repopulating the GALT than mice that have Peyer's patches anlagen.

To enrich for human cells of lymphoid origin in the rectal mucosa in our humanized mouse model, we induced local inflammation. Mucosal inflammation as seen in sexually transmitted diseases is a risk factor for HIV acquisition be increasing numbers of HIV target cells and disturbing epithelial integrity[41]. We treated humanized mice with Il-1β rectally 24 hours before HIV challenge. In untreated mice no infections could be seen, in treated mice 1/17 showed a detectable plasma viral load. Immune histochemical analysis of Il-1β-treated uninfected mice suggests that, despite an inflammatory response and cell infiltration, human cell numbers were probably still too low to establish infection. It is believed that HIV first

infects a local founder population in the lamina propria of the mucosal tissue and amplifies there before systemic seeding and dissemination of the infection[100]. In our model, local expansion of infection is difficult with only a few human cells present in the lamina propria. We only observed disseminated infection when mice were injected intraperitoneally with HIV, and the mucosal amplification step was not needed.

To attract human cells more efficiently to the GALT, we treated humanized mice with DSS, a compound widely used in murine models of inflammatory bowel disease. Repeated treatment with DSS leads to a chronic colitis with infiltration of lymphocytes and macrophages[86]. After three cycles of DSS treatment of humanized mice, we also saw infiltration of human cells into the rectal mucosa. Moreover, we confirmed colitis induction by following weight changes and finally by histological scoring of rectal tissue. Mice tolerated treatment well up to 1 week after the last DSS administration when a rapid decline in health occurred and half of the mice had to be euthanized. The HIV challenge could be a reason for the observed health decline. However, all mice were similarly affected, even surviving mice, which remained HIV negative. Notably, in non-DSS-treated mice, the same HIV challenge never elicited any symptoms. We therefore conclude that the DSS chronic colitis model is not suitable for rendering humanized mice permissive to rectal challenges with HIV.

It is still unknown whether cell-free or cell-associated HIV is preferably transmitted. To develop microbicides or vaccines, it is essential to know whether protection is needed against free virions, infected cells, or both. In simian and feline models, both cell-free and cell-associated virus transmission can be observed with different efficiencies, depending on experimental design[38,101,102]. Results from studies in humans are conflicting: both free virions and infected cells are detected in cervicovaginal fluid[103] and semen[104]. In cervical explants, which frequently are used for pre-clinical microbicide testing, cell-free and cell-associated HIV are infectious[105]. Here, we detected only minimal transmigration of rectally applied mononuclear cells into the mucosa both in untreated and Il-1β-treated humanized mice. Further infection experiments with cell-associated HIV confirmed this observation. None of the mice exposed to HIV-infected PBMC showed systemic viral replication, not even mice that had DSS colitis. Thus, cell-associated HIV transmission is, at least in our model, not more efficient than cell-free HIV transmission.

HIV strains with selective co-receptor use or even more subtle viral variants may differ in their ability to establish infection by the mucosal route. In humans CCR5-tropic HIV is transmitted preferably over CXCR4-tropic HIV[106] and even in the group of CCR5 viruses,

potential for transmission is diverse. Patients during acute infection show a more homogenous viral population, whereas patients in the chronic phase harbor many distinct variants[107,108], indicating that only some of the viral variants in the transmitter are passed on. However, the characteristics of HIV variants preferentially transmitted are unknown so far. The CCR5-tropic HIV variant YU-2, which we used in our study for rectal challenge, was first isolated from neural tissue of a child suffering from AIDS[109]. There is some uncertainty whether it is easily transmitted by the mucosal route or not. In any case, YU-2 replicates well in humanized mice and establishes disseminated infection after intraperitoneal injection[68], and the three other HIV strains we tested in this study, were not more efficient in rectal HIV transmission. The other two studies[66,84] showing mucosal HIV transmission in humanized mice showed that the mice were permissive to CXCR4-tropic HIV infection by the rectal route. However, Sun et al. challenged mice with HIV after mechanical disruption of the epithelial layer. It remains unknown whether the CXCR4-tropic viral strain used would have been transmitted otherwise. So far, we do not know whether the same bottleneck seen in humans for mucosal HIV transmission exists in humanized mice.

In conclusion, our data indicate that GALT reconstitution in $RAG2^{-/-}c_{\gamma}^{-/-}$ mice transplanted with $CD34^{+}$ cells from cord blood is low and these mice seem to be quite resistant to rectal HIV transmission, even in an inflammatory setting. Their value to study measures preventing mucosal HIV transmission probably is limited. Further efforts are needed to clarify which mouse strain and transplantation protocol are best suited to generate the optimal humanized mouse.

Acknowledgements

This work was supported by amfAR grant 106762-41-RGMT. RFS is supported by the Swiss National Science Foundation and the Baugarten Stiftung, SB is financially supported by EMPIRIS Foundation, Zurich, Switzerland. We thank the staff of the Maternité Triemli (Zurich, Switzerland) for cord blood collection, M. Ito (Central Institute for Experimental Animals, Kawasaki, Japan) for providing the original RAG2$^{-/-}$c$_{\gamma}^{-/-}$ mice, the staff of S. Regenass (Division of Clinical Immunology, University Hospital Zurich, Switzerland) for measuring plasma viral load, Roche (Basel, Switzerland) for providing HIV RT-PCR reagents, P. Vernazza (Kantonsspital, St.Gallen, Switzerland) for providing seminal plasma samples, N. Corazza (Institute of Pathology, Division of Immunopathology, University of Bern, Switzerland) for discussion of protocols and data of intestinal lymphocyte isolation, F. Burgener (Division of Infectious Diseases and Hospital Epidemiology, University Hospital, Zurich) for technical help with cryo-sections, the staff at the animal facilities of the University Hospital Zurich and the University Irchel, Zurich, Switzerland, L. Bestmann and M. Hersberger (Division of Clinical Chemistry, University Hospital Zurich, Switzerland) for analysis of seminal plasma, and Hugo Stocker (ETH, Zurich, Switzerland) for carefully reading the manuscript.

Contribution

UH designed, conducted, and analyzed all experiments. SB, ES, and NG assisted in some experiments. MH helped with immunohistochemistry and scientific input. TB helped to design and to analyze the GALT engraftment control and the DSS model. SR measured plasma viral load. RS designed and supervised the experiments. UH and RS wrote the paper.

3. Pathogenesis mechanisms of bacterial translocation in HIV infection

Published as Hofer et al., Semin Immunopathol. 2009 Jul;31(2):257-66.

Disturbance of the gut-associated lymphoid tissue is associated with disease progression in chronic HIV infection

Ursula Hofer, Roberto F Speck

Division of Infectious Diseases and Hospital Epidemiology, University Hospital Zurich, University of Zurich, 8091 Zurich, Switzerland

Why and how HIV makes people sick is highly debated. Recent evidence implicates heightened immune activation due to breakdown of the gastrointestinal barrier as a determining factor of lentiviral pathogenesis. HIV-mediated loss of Th17 cells from the gut-associated lymphoid tissue (GALT) impairs mucosal integrity and innate defense mechanisms against gut microbes. Translocation of microbial products from the gut, in turn, correlates with increased immune activation in chronic HIV infection and may further damage the immune system by increasing viral and activation-induced T-cell death, by reducing T-cell reconstitution due to tissue scarring, and by impairing the function of other cell types, such as γδ T cells and epithelial cells. Maintaining a healthy GALT may be the key to reducing the pathogenic potential of HIV.

Introduction

HIV infection is characterized by a progressive immunodeficiency, which is reflected in a steady decline of $CD4^+$ T cells. Disease progression varies considerably from patient to patient. Some develop the acquired immunodeficiency syndrome (AIDS) after a few months; others remain healthy for decades. Such a disparity also exists in monkeys of distinct species infected with simian immunodeficiency virus (SIV). SIV infection of African monkeys, the natural hosts for SIV, does not result in simian AIDS, but SIV infection of most Asian monkeys rapidly progresses to AIDS[110]. Remarkably, despite 25 years of HIV and SIV research, the reasons for these differences in disease outcome are only partially understood. To design new treatment approaches, including vaccination or eradication strategies, we need to understand the basis for these disparities.

Immune dysfunction and disease onset cannot be explained solely by the direct cytopathic effect of the virus. Other factors have been implicated in T-cell loss, such as the killing of infected cells by cytotoxic T cells, bystander death of uninfected cells due to HIV products, and T-cell dysfunction and death due to heightened levels of immune activation[73]. A hyperactive immune state with high T-cell turnover, polyclonal activation of B cells, and elevated pro-inflammatory cytokines is characteristic of HIV infection[73]. In fact, the activation status of CD8+ T cells is one of the best predictors of disease progression[72]. While the association of immune activation and HIV infection is well known, its causes are only partially understood.

A recent model links disturbance of the gut-associated lymphoid tissue (GALT) to immune activation and lentiviral disease progression[51]. The GALT is one of the primary organs affected by HIV and SIV, and GALT lymphocytes are the primary target cells of HIV during mucosal transmission. After rectal exposure, they are the first immune cells that encounter HIV. For infections by different routes, they form a large pool of HIV target cells, in which HIV efficiently spreads and replicates. Later on, during chronic HIV infection, loss of GALT integrity may have a major impact on AIDS pathogenesis[111]. Briefly, during the chronic phase of infection, elevated levels of microbial products are found in the systemic circulation, and these levels correlate with immune activation and disease state. Initial studies looking at interactions of lentiviruses with the GALT focused mainly on numbers of infected and dying T cells. Recently, studies have proposed cellular and molecular mechanisms that clarify the intricate role of GALT in HIV pathogenesis. Here we will review these studies and relate their findings to lentiviral disease progression.

Profound depletion of GALT lymphocytes occurs during acute lentiviral infection

The CD4+ T-cell count in the blood is a commonly used clinical marker for monitoring progression rate in HIV infection. In acute HIV infection, blood CD4+ T-cell numbers may decline sharply, but as soon as an antiviral immune response is established, blood CD4+ T cells have the potential to recover. Subsequently, during chronic HIV infection, blood CD4+ T-cell counts decline slowly, but this loss can be partially reversed by successful antiretroviral treatment. However, T cells in the GALT display entirely different kinetics: in SIV-infected macaques and in HIV-infected humans, CD4+ T cells of the lamina propria are rapidly and profoundly depleted in the first days after infection, and their numbers remain low throughout the course of disease[8,112,113]. Even after years of antiretroviral therapy, this loss is only partially reversible in most patients[114,115]. Thus, lymphocyte depletion in the GALT is a distinct feature of lentiviral infection.

The pronounced loss of GALT lymphocytes probably occurs because the majority of newly transmitted HIV strains use CCR5 as a co-receptor for cell-entry[20]. A high percentage of CD4+ T cells in the intestinal effector sites are CCR5 positive memory cells, and thus, GALT lymphocytes are ideal viral targets. In contrast, many T cells in blood and lymph nodes are naive and hence CCR5 negative[116]. During acute SIV infection, up to 60% of all memory CD4+ T cells in the jejunum harbor HIV DNA[23], suggesting that direct viral cytopathic effects cause the massive loss of CD4+ T cells during acute infection. Furthermore, high levels of Fas-mediated apoptosis of infected and uninfected CD4+ T cells occurs during peak viremia[117]. Direct viral killing and bystander death of uninfected cells wipe out most of the CD4+ memory cell population in the GALT.

When the loss of GALT lymphocytes was first discovered, it appeared plausible that this defect in mucosal immune surveillance would have dire consequences for the whole immune system and would influence disease progression. Surprisingly, however, SIV depletes lamina propria T cells in all monkey species, irrespective of whether they will develop simian AIDS or not. Natural SIV hosts, such as sooty mangabeys and African green monkeys, do not progress to AIDS despite high viral loads, but rhesus macaques infected with the same SIV strain show blood CD4+ T-cell loss and disease progression, closely resembling HIV pathogenesis in humans[110]. In both the pathogenic and non-pathogenic SIV model, GALT CD4+ T cells are lost at similar rates during acute infection[57,118]. At later times, however, the GALT can be partially restored in the non-pathogenic models. These observations indicate that acute intestinal CD4+ T-cell loss is a basic feature of lentiviral infections but is not

predictive of later disease outcome. Low numbers of GALT lymphocytes per se do not make animals or humans sick; additional factors are important for the pathogenic potential of HIV.

In chronic lentiviral infection, circulating microbial products are associated with immune activation

Differences in pathogenic and non-pathogenic SIV models become more apparent when GALT function, rather than simple lymphocyte numbers, is considered. The GALT prevents microbial invasions from the intestinal lumen. In chronic lentiviral infections, this function seems to be disturbed, and more microbial products are found in the systemic circulation.

Brenchely et al.[119] measured plasma levels of lipopolysaccharide (LPS), a component of gram-negative bacterial cell walls, as an indicator of microbial translocation from the gut. They found higher plasma LPS levels in chronically HIV-infected patients than in healthy subjects. Notably, a similar LPS increase was observed in the pathogenic SIV rhesus macaque model. In contrast, in the non-pathogenic SIV sooty mangabey model, LPS levels were low, irrespective of SIV status. Thus, in SIV-infected sooty mangabeys, GALT immune function appears to be sufficient to prevent increases of bacterial products in the systemic circulation despite intestinal CD4+ T-cell depletion.

To further elucidate the connection between clinical outcome and microbial translocation, plasma LPS levels were examined in defined subsets of patients. For example, Hunt et al.[120] verified a finding from Brenchely et al.[119]: HIV-infected elite controllers—patients with undetectable viral loads without antiretroviral treatment—have LPS levels similar to patients with higher viral loads. At first glance, these data might indicate that no connection between plasma LPS and disease outcome exists. However, Hunt et al. showed that plasma LPS in elite controllers correlated with immune activation, which was associated with lower CD4+ T-cell counts. This finding suggests that, in elite controllers, immune activation is detrimental and leads to CD4+ T-cell loss, despite suppressed plasma viral load, and that bacterial translocation may be the activating factor.

Marchetti et al.[121] investigated bacterial translocation in patients on highly active antiretroviral therapy (HAART). All patients on HAART had lower plasma levels of LPS than untreated control patients. As defined by blood CD4+ T-cell reconstitution, therapy success was associated with even lower LPS levels. Patients who did not show T-cell recovery despite suppressed HIV RNA had more bacterial translocation as quantified by plasma LPS than patients who responded immunologically and virologically to HAART. This means that in both groups with suppressed viral loads—either in elite controllers or in HAART treated patients—low-level microbial translocation still occurs and seems to be associated with lower CD4+ T-cell counts.

In support of the link between bacterial translocation and HIV pathogenesis, Ancuta et al.[122] found higher plasma LPS levels in patients with HIV-associated dementia than in HIV-positive patients without neurocognitive impairment. They proposed LPS-mediated monocyte activation and trafficking to the brain as the underlying mechanism of this association between LPS and dementia. As supporting evidence, they showed up-regulation of activation markers on monocytes and increases of soluble CD14, IL-6, and CCL2 in the plasma of HIV-infected patients.

To further investigate the effects of bacterial translocation on immune activation, Gregson et al.[123] compared plasma LPS levels in HIV-infected individuals to HIV-negative patients with active colitis, either colitis ulcerosa or Morbus Crohn. All groups had similarly elevated LPS levels. However, colitis usually shows a fluctuating course: the gastrointestinal barrier, in fact, is only affected during an inflammatory phase but is relatively intact between flare-ups. In contrast, HIV-infected patients seem to experience constant bacterial translocation and immune activation.

However, factors other than bacterial translocation are thought to contribute to sustained immune activation. In particular, Gregson et al. reported that the activated phenotype of NK cells seen in their cohort of HIV-positive patients was linked to the plasma viral load and not to plasma LPS. Strikingly, the colitis patients, which had LPS levels as high as the HIV-positive patients, showed only low numbers of activated CD8+ T cells, indicating that high levels of plasma LPS alone do not cause activation of CD8+ T cells.

A study in patients undergoing interruption of antiretroviral therapy[124] supports the notion that CD8+ T-cell activation not only depends on plasma LPS. In the first 6 weeks after stopping HAART, LPS levels remained unchanged, whereas percentages of activated blood CD8+ T cells increased. Only later did plasma LPS levels rise, but still no association with activated CD8+ T cells was detected, perhaps because the study examined a relatively small population. Interestingly, the delayed onset of LPS increase after treatment interruption mirrors the initial findings of Brenchely et al.[119], which also showed that, for LPS to increase, some time of sustained viral replication was required. Plasma LPS levels were similar in patients with acute HIV infection and in healthy control subjects. A possible explanation is that, initially, systemically circulating LPS can be cleared, but over time, this function is impaired. The treatment interruption study showed a negative correlation between changes of plasma LPS levels and endotoxin core antibodies (EndoCAb) in the early phase, when plasma LPS levels are controlled. Later on, this correlation is lost. They claimed that, initially, LPS is cleared by

EndoCAb, and EndoCAb levels therefore decrease. After some time, clearance is no longer effective, due either to excessive microbial translocation or to inadequate B-cell function.

In conclusion, there is universal agreement that heightened levels of circulating bacterial products correlate in general with immune activation in pathogenic lentiviral infections (Table 1). However, careful interpretation is needed when deducing the mechanistic links between these events. A direct causative relation has not been established between heightened LPS levels and HIV disease progression. In an alternative explanation, microbial translocation may be an epiphenomenon of an activated and dysfunctional immune system.

Also what is the deficit in the GALT resulting in bacterial translocation? Recent studies propose a mechanism linking depletion of a subset of GALT effector site lymphocytes, namely Th17 cells, with breakdown of the gastrointestinal barrier. Such a link is a first step in explaining the relationships between HIV infection and GALT dysfunction.

Table 1. Bacterial translocation in lentiviral infections.

Study	Subjects	Parameters	Results
Ancuta et al.[122]	HIV in humans: progressors with or without HIV-associated dementia	LPS sCD14, LBP, EndoCAb, IL-6, CCL2 CD16+, CD69+, or CCR5+ monocytes	higher LPS levels in patients with dementia, association between monocyte activation, LPS and dementia
Brenchley et al.[119]	HIV in humans: negative, acute/early, chronic, AIDS	LPS sCD14, LBP, EndoCAb LPS reactivity of monocytes ex vivo plasma IFNα CD8+ HLA DR+ CD38+ T-cells	higher LPS levels in chronic infection, correlation with immune activation
	HIV in humans: negative, before and after HAART		LPS decrease under HAART, but no normalisation
	HIV in humans: negative, controllers, progressors		higher LPS levels in controllers compared to uninfected subjects, no significant difference to progressors
	SIV in rhesus macaques: negative, positive, positive treated with antibiotics		LPS increase after SIV infection in RMs, reduction under antibiotics
	SIV in sooty mangabeys: negative, positive		low LPS levels in both groups of SMs
Gregson et al.[123]	HIV in humans: negative, untreated/viremic, HAART Colitis: active Crohn's disease or colitis ulcerosa	LPS CD8+ HLA DR+ CD38+ T-cells NK cell activation LPS reactivity of NK cells ex vivo	higher LPS levels in HIV infected, irrespective of HAART no difference of LPS levels between HIV infected and colitis patients NK and CD8 T-cell activation in HIV infected patients, not in colitis patients
Hunt et al.[120]	HIV in humans: negative, controllers, progressors	LPS CD8+ HLA DR+ CD38+ T-cells	higher LPS levels in controllers compared to uninfected subjects, no significant difference to progressors, correlation between activated CD8 T cells and LPS in controllers
Marchetti et al.[121]	HIV in humans: untreated/advanced, HAART/full responders, HAART/immunological non responders	LPS, bacterial 16sRNA CD4 and CD8 Ki67+	treatment reduced LPS levels overall, but immunological non responders had higher levels than full responders
Papasavvas et al.[124]	HIV in humans: HAART before and after short/long time treatment interruption	LPS sCD14, LBP, EndoCAb CD8+ CD38+ T-cells	no LPS increase after short time treatment interruption, but already increase of CD8+ T-cell activation after long time treatment interruption LPS increase

Th17 cells help are essential for the integrity of the gastrointestinal barrier

Th17 cells are important for intestinal homeostasis (reviewed in[125]). Briefly, this subset of CD4+ T cells is characterized by the production of IL-17 in response to stimulation, but they also secrete other cytokines, including TNF-α, IL-1, IL-6, IL-21, and IL-22. Their strong pro-inflammatory properties are both beneficial and harmful. Initial animal studies identified Th17 cells as important mediators of autoimmune disease and tissue damage[126,127], yet with their ability to recruit neutrophils[128], they also are important for controlling infections by bacteria and fungi[129-134]. Furthermore, Th17 cells are involved in epithelial regeneration[135]. They stimulate production of defensins and mucin[136,137], and they induce the expression of claudins[138], which are components of epithelial tight junctions. Finally, IL-22, an important Th17 cytokine, increases the production of LPS binding protein (LBP) in the liver[139]. Considering the massive CD4+ T-cell depletion in the lamina propria after HIV infection, it is reasonable to assume that Th17 cells are also depleted by HIV. And with their multiple functions in controlling epithelial integrity and microbial invasion, their loss likely affects the integrity of the gastrointestinal barrier.

While Th17 cells are permissive to HIV *in vitro*, they do not appear to be the preferential targets of HIV[140] nor of SIV[141]. Infection rates are similar in all CD4+ T cells, irrespective of Th17 or Th1 differentiation. Nevertheless, in acutely SIV-infected rhesus macaques, the intestinal Th17 responses seem to be afflicted more than the Th1 response. Checchinato et al.[141] found that the percentage of IL-17-producing cells, as related to all intestinal CD4+ T cells, was much lower, whereas the percentage of IFN-γ producing cells was much greater than in healthy animals. Relative numbers, however, do not take into account the massive reduction of absolute CD4+ T-cell numbers in the gut during acute SIV infection. Even though relative numbers of Th1 cells may be increased, the absolute numbers are still strongly reduced. We can only poorly judge the importance of relative differences between Th17 and Th1 responses in light of the overall loss of lymphocytes.

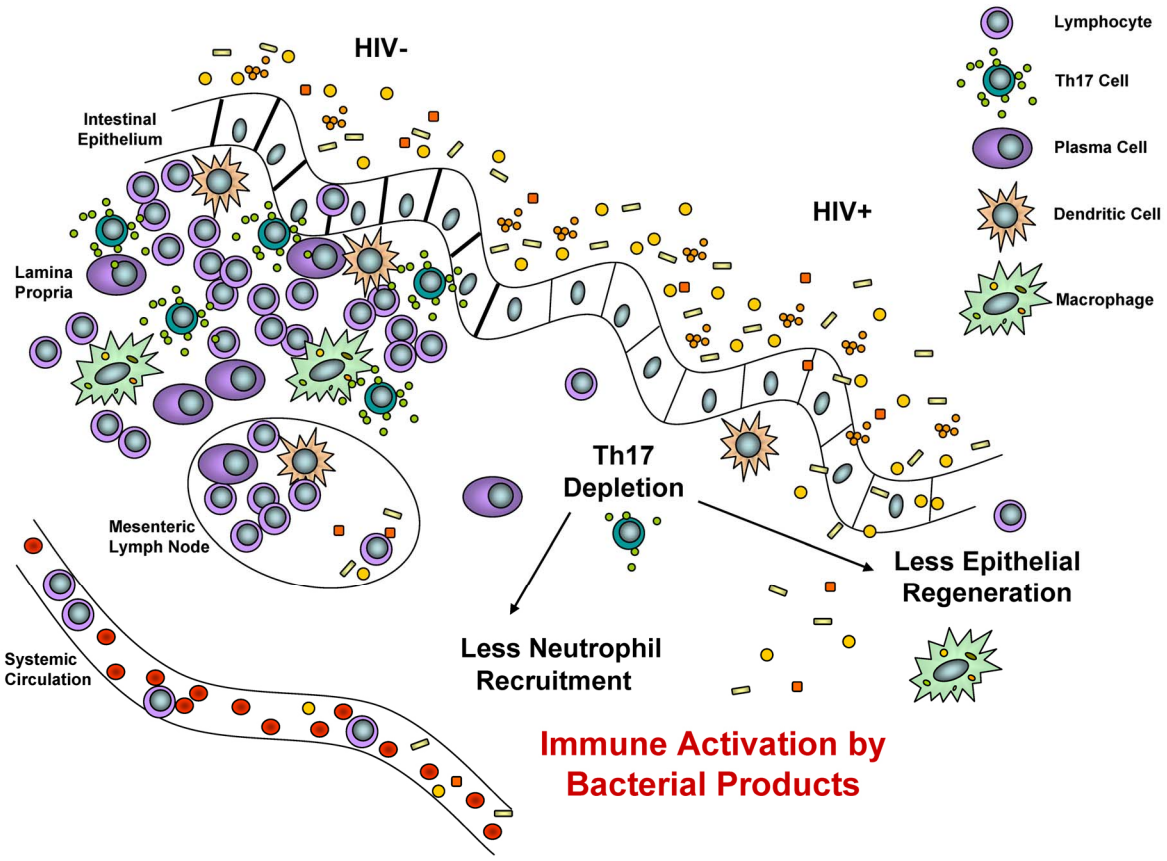


Figure 1. HIV infection depletes lymphocytes, including Th17 cells, from the GALT effector sites. Th17 cells are essential in maintaining an intact gastrointestinal barrier against gut microbes by producing cytokines that are essential for epithelial regeneration and neutrophil recruitment. In HIV-positive individuals, circulating microbial products can be detected, and these levels correlate with immune activation.

Brenchley et al.[140] further investigated the effects of HIV infection on T-cell subsets at different mucosal surfaces and compared T-cell responses in lung and gut. In contrast to results from bronchoalveolar lavage samples, they found a preferential depletion of IL-17-producing CD4+ T cells in the gut of HIV-infected humans. This result indicates that HIV infection especially reduces the intestinal Th17 function. In contrast to reports in humans, in SIV-infected sooty mangabeys the relative numbers of IL-17+ CD4+ T cells in the gut are similar to those in uninfected animals. The authors linked this intact Th17 function in SIV-infected sooty mangabeys with their non-pathogenic phenotype. Again, one should be careful in interpreting relative cell numbers from the gut, because of the overall depletion of CD4+ T cells.

Still, the data from both these studies suggest that the intestinal Th17 response is diminished during lentiviral infection, probably due to depletion or dysfunction of these cells. What are

now the consequences of an impaired Th17 response in the gut? Raffatellu et al.[142] addressed this question in an elegant study using a gut ligation model in rhesus macaques to study *Salmonella* translocation. Individual intestinal loops from the same animal are either mock inoculated or exposed to *Salmonella*, and immune responses then can be quantified. Intestinal *Salmonella* inoculation induced a strong mucosal Th17 response in this model. This Th17 response, however, was blunted when the rhesus macaques were chronically SIV infected. Consequently, SIV infected rhesus macaques had a greater degree of *Salmonella* translocation to mesenteric lymph nodes than SIV-negative monkeys. One could argue that this effect of the SIV infection on immune control of *Salmonella* translocation may not be Th17 specific but rather is due to overall GALT depletion. But Raffatellu et al. confirmed their data in mice with a targeted defect in the IL-17 receptor. Due to abrogated IL-17 signaling, these mice had less production of other IL-17 dependent cytokines upon gastrointestinal *Salmonella* inoculation, they recruited less neutrophils to the mucosa, and they were unable to control *Salmonella* translocation. The data from Raffatellu et al. corroborate the current view that HIV infection results in a loss of the intestinal Th17 response and that this affects the integrity of the gastrointestinal barrier.

In conclusion, these studies propose a model in which HIV/SIV-mediated Th17 depletion from GALT effector sites impairs the gastrointestinal barrier. This, in turn, leads to translocation of intestinal microbes or microbial products, which then contribute to immune activation (Fig. 1). However, we must be aware that no definitive experimental proof links Th17 dysfunction and elevated LPS levels. Increased intestinal *Salmonella* invasion after SIV infection is an intriguing hint but not the same as continuous translocation of harmless commensal bacteria. In Cecchinato et al.[141], for example, no correlation between LPS levels and GALT Th17 cell numbers was found in SIV-infected macaques. Nevertheless, Th17 cells probably are important in overall immunodeficiency. Recently, a defect in Th17 differentiation in patients with autosomal dominant hyper Ig-E syndrome[143] was identified. Hyper-Ig-E syndrome is characterized by *Candida* infections, recurring pneumonia, skin abscesses, and lymphomas—a clinical picture reminiscent of AIDS. However, no data exist on the integrity of the gastrointestinal barrier and bacterial translocation in these patients.

A disturbed gastrointestinal barrier may have negative effects on the immune system

While circulating microbial products correlate well with activation markers on immune cells, the exact connection between these two phenomena is not clear. Does immune activation render the immune system so dysfunctional that it can no longer control bacterial translocation, or do the circulating bacteria and bacterial components activate the immune system and thereby contribute to the progressive immunodeficiency observed in HIV? Both possibilities likely co-exist, leading to a vicious circle in which one factor triggers the other one. In any case, sustained immune activation has negative effects on T-cell function and survival. For one, sustained activation provides a large pool of activated CD4+ T cells, which are optimal viral targets since HIV more efficiently infects and replicates in activated CD4+ T cells[144]. However, in most of the above studies, no positive association between plasma viral load and LPS was found[119,124]. This is not surprising, since many other factors besides overall activation status can influence viral load (e.g., HIV-specific immune responses, chemokine levels, and viral fitness).

In addition to providing a continuous reservoir of optimally activated HIV target cells, microbial products may initiate activation-induced cell death. An innovative study by Bourgeois et al.[145] in mice showed that naive CD4+ T cells are especially affected by activation-induced cell death from gut antigens. While investigating peripheral T-cell dynamics after thymic ablation, they found a substantial decay in naive CD4+ T cells, while CD8+ T-cell counts remained relatively stable. As the cause of CD4+ T-cell activation and subsequent activation-induced death of naive cells, they identified translocation of microbial products from the gut. Indeed, the mice had higher levels of LBP, an acute phase protein produced in the liver after exposure to LPS. Notably, in transfer experiments, they established that those CD4+ T cells reactive to gut microbes and involved in intestinal inflammation were lost specifically. Induction of intestinal inflammation by transfer of T cells reactive to gut microbes is an established tool in colitis research[146]. Naive CD4+ T cells depleted of CD25high cells are transferred into immunodeficient mice, there they proliferate and respond to intestinal antigens. Bourgeois et al.[145] found that CD4+ T cells from donor mice with bacterial translocation could no longer induce colitis in the recipient mice. Because of the antigen recognition and activation mechanisms in CD4+ T cells, these cells may be especially prone to effects from microbial translocation. Microbial products circulate in the extracellular compartment and are taken up by antigen-presenting cells for processing and presentation on

MHC class II molecules. Thereby, CD4+ T cells (i.e., those cells recognizing antigen in the context of MHC II) but not CD8+ T cells are activated and die.

HIV infection likely influences other immune cells in the GALT. For example, T-regulatory cells (Tregs) are also affected. Tregs are abundant in the intestinal mucosa and are involved in maintaining a healthy balance between tolerance and immune control of gut microbes. Highly viremic SIV-infected macaques have more mucosal Tregs than animals with low viral loads[147]. These numerous Tregs may contribute to a less efficient SIV-specific immune response and consecutively to increased SIV loads. However, this hypothesis is controversial. Another study showed that inhibiting Treg function with a blocking antibody to CTLA-4 increased SIV replication, particularly at mucosal sites[148]. This study suggests that Tregs are protective by reducing immune activation and viral replication. Thus, increased Treg numbers in highly viremic macaques may represent a negative regulatory feedback mechanism.

The GALT contains many cell types besides classical CD4+ T cells (e.g., plasma cells, dendritic cells, monocytes, or γδ T cells). Little is known about their fates during chronic lentiviral infection. Some studies suggest that they may be essential for limiting microbial translocation. In addition to depletion of Th17 cells, Brenchley et al.[140] also noted loss of myelomonocytic cells in the GALT of HIV-infected individuals. These myelomonocytic cells, which include granulocytes, macrophages, and dendritic cells, are essential for killing and phagocytosis of gut microbes, and for orchestrating an adaptive immune response. Another hint for the involvement of additional GALT cells in controlling microbial translocation comes from one of the non-pathogenic SIV models: Sooty mangabeys, which do not show prolonged systemic LPS increases despite GALT CD4+ T-cell depletion, have astoundingly high numbers of γδ T cells. γδ T cells build an interface between innate and adaptive immunity and are essential for immune function at mucosal surfaces[149]. Therefore, they may have a protective effect on integrity of the intestinal barrier and may limit bacterial translocation. Compared to humans, sooty mangabeys have higher numbers of γδ T cells, and their γδ T-cell response to bacterial antigens is even enhanced[150] after SIV infection. In contrast, in HIV-infected humans γδ T-cell counts are lower, and the cells are anergic to stimulation with mycobacterial antigens[151].

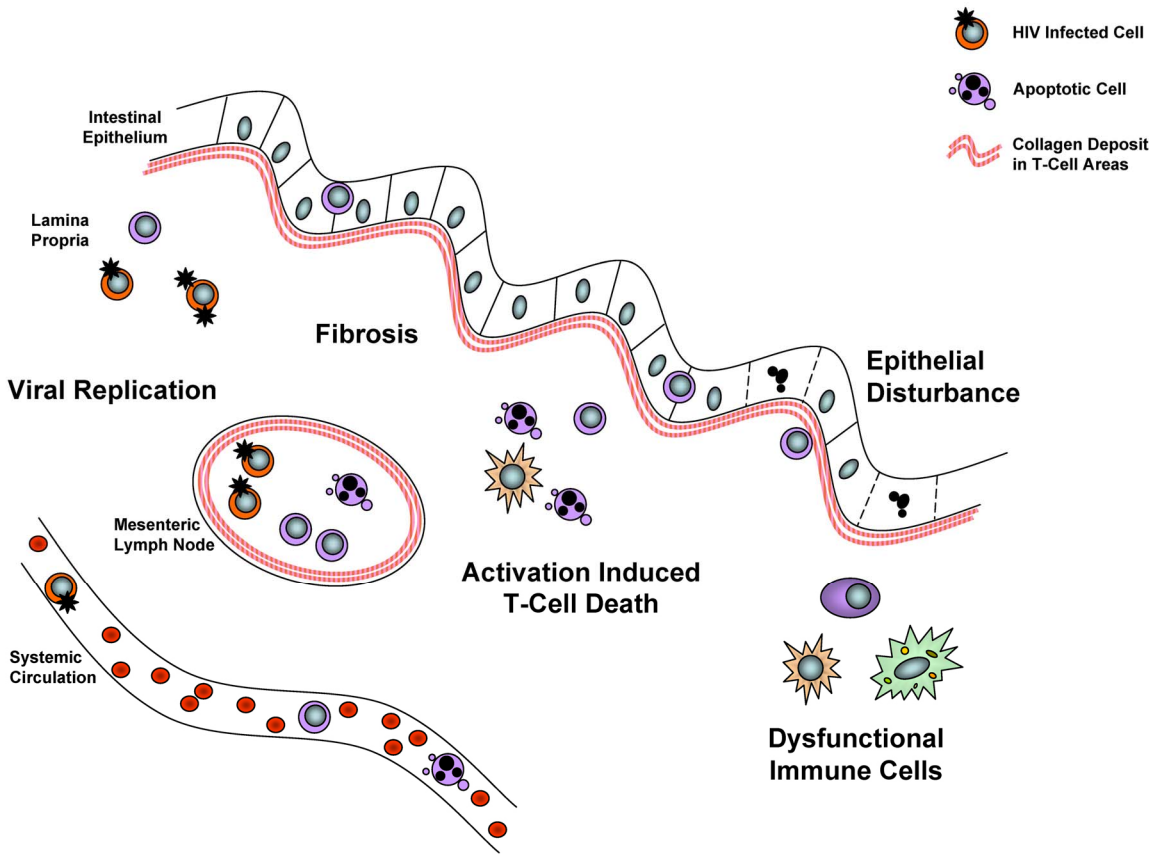


Figure 2. Microbial translocation and ensuing immune activation can damage the immune system and contribute to HIV pathogenesis. By increasing numbers of activated cells, which are preferentially infected, microbial translocation can increase viral replication. Furthermore, activated cells are prone to activation-induced cell death, and immune reconstitution is limited due to fibrosis of the lymphatic tissue. Other immune cells, such as macrophages or γδ T cells, also are negatively affected by lentiviral infection and may be dysfunctional. Epithelial disturbance is also observed in HIV infection.

HIV infection also interferes with stromal and epithelial cells of the intestinal mucosa. During acute SIV infection massive apoptosis occurs in the gut epithelium[152]. Pro-inflammatory genes are up-regulated, and genes responsible for epithelial regeneration and digestive/metabolic functions are down-regulated[153]. Notably, in chronic infection, a pro-inflammatory milieu marked by up-regulation of IL-6 and STAT3 persists[154], and mucosal IL-2, IL-4 and TNF-α are increased, leading to increased epithelial permeability[155]. Intestinal inflammation and epithelial apoptosis not only impairs the gastrointestinal barrier, chronic inflammation also has negative effects on overall tissue architecture. GALT tissue from HIV-infected patients shows marked fibrosis, and the amount of collagen deposition is even higher than in other lymphatic organs[156]. In lymph nodes, intense fibrosis correlates with low CD4+ T-cell counts and poor T-cell reconstitution under HAART[157,158], and the same very likely

applies to GALT fibrosis. This observation explains the slow and poor reconstitution of GALT lymphocytes under HAART, despite suppression of the local mucosal inflammation and permeability changes mediated by successful antiretroviral treatment[155].

In summary, accumulating data indicate that HIV causes a profound and complex disturbance of the mucosal immune function. The deleterious effects of HIV are not limited to CD4+ T cells. In chronic HIV infection, disturbed GALT function and microbial translocation are accompanied by an incessant vicious circle of immune activation and inflammation with deleterious consequences on viral replication, T-cell and epithelial cell death, and dysfunction of multiple additional cells (Fig. 2).

Conclusions

The GALT is one of the major organs affected by HIV infection. Viral replication and T-cell loss are even more pronounced in the intestinal lamina propria than in other lymphoid tissues. Furthermore, in chronic HIV infection, a poorly controlled translocation of bacterial products (e.g., LPS) occurs and correlates with immune activation markers, which in turn are associated with disease progression. Recently, a handful of studies identified the critical role of Th17 cells in this process. During pathogenic lentiviral infections, Th17 function in the GALT is reduced and invasion of gut bacteria—directly shown so far only with *Salmonella*—is increased.

However, many questions remain. Cause and effect relations in this circle of immune dysfunction and bacterial translocation are difficult to pin down. Consequently, one cannot predict if reduction of bacterial translocation or immune activation would be a beneficial therapeutic approach. Studies with compounds blocking immune activation so far did not show positive effects[159,160], maybe due to a general immunosuppressive effect of these drugs. Despite reduced immune activation, which may prevent further deterioration of immune functions, such drugs will also reduce immune responses directed against the virus. The optimal therapy should reduce the damaging general activation of immune cells, boost HIV-specific immune responses, and activate antimicrobial defense at mucosal surfaces to reduce translocation of gut microbes.

Acknowledgment

UH is supported by the Swiss National Science Foundation (SNSF) with a scholarship 323530-123717. RFS is supported by the SNSF (#31-118391/1).

4. Bacterial translocation and immune activation in HIV infected humanized mice

Accepted as Hofer et al., PLoS Pathog. March 2010.

Inadequate clearance of translocated bacterial products in HIV-infected humanized mice

Ursula Hofer[1], Erika Schlaepfer[1], Stefan Baenziger[1], Marc Nischang[1], Stephan Regenass[2], Reto Schwendener[3], Werner Kempf[4], David Nadal[5], Roberto F Speck[1]

[1]Division of Infectious Diseases and Hospital Epidemiology, and [2]Division of Clinical Immunology, [3]Institute of Molecular Cancer Research, [4]Kempf and Pfaltz Histological Diagnostics, [5]Experimental Infectious Diseases and Cancer Research, Division of Infectious Diseases and Hospital Epidemiology, University Children's Hospital Zurich, University of Zurich, Zurich, Switzerland

Bacterial translocation from the gut and subsequent immune activation are hallmarks of HIV infection and are thought to determine disease progression. Intestinal barrier integrity is impaired early in acute retroviral infection, but levels of plasma lipopolysaccharide (LPS), a marker of bacterial translocation, increase only later. We examined humanized mice infected with HIV to determine if disruption of the intestinal barrier alone is responsible for elevated levels of LPS and if bacterial translocation increases immune activation. Treating uninfected mice with dextran sodium sulfate (DSS) induced bacterial translocation, but did not result in elevated plasma LPS levels. DSS-induced translocation provoked LPS elevation only when phagocytic cells were depleted with clodronate liposomes (clodrolip). Macrophages of DSS-treated, HIV-negative mice phagocytosed more LPS ex vivo than those of control mice. In HIV-infected mice, however, LPS phagocytosis was insufficient to clear the translocated LPS. These conditions allowed higher levels of plasma LPS and CD8+ cell activation, which were associated with lower CD4+/CD8+ cell ratios and higher viral loads. LPS levels reflect both intestinal barrier and LPS clearance. Macrophages are essential in controlling systemic bacterial translocation, and this function might be hindered in chronic HIV infection.

Summary

HIV infection leads to continuous destruction of the body's immune defenses. Furthermore, disease progression is linked to heightened levels of immune activation. However, the underlying activating factors and their relationships to HIV pathogenesis are controversial. In patients with chronic HIV infection, bacteria and their products, such as lipopolysaccharide (LPS), translocate from the intestinal lumen into the systemic circulation. In the current study, we investigated the pathogenic potential of bacterial translocation in HIV-infected humanized mice. By modulating the amount of bacterial translocation in the mice, we determined that LPS elevation depends on intestinal barrier dysfunction and defective LPS clearance by macrophages. HIV-infected mice showed inadequate LPS clearance, leading to a cascade of uncontrolled bacterial translocation, T-cell activation, HIV replication, and T-cell loss. Our study highlights how important the interplay between different immune cells is for maintaining a healthy balance between immune activation with the goal to defend the body against microbes and detrimental activation that fuels HIV replication.

Introduction

The clinical course of HIV infection varies considerably among patients, and the variability is even greater in simian models. Asian monkeys infected with simian immunodeficiency virus (SIV) rapidly progress to AIDS, but African monkeys do not get sick[110]. In general, a pathogenic course of retroviral infection is characterized by high levels of immune activation[161], and bacterial translocation from the intestinal tract has been implicated as an underlying activating mechanism[56,120-124]. The integrity of the intestinal barrier is impaired early in acute retroviral infections[162], and a substantial fraction of the intestinal CD4+ T cells are lost within days after infection[8,112].

However, bacterial translocation manifests itself only later. Low T-cell numbers in the gut are an important characteristic of HIV or SIV pathogenesis[111,163], but intestinal CD4+ T-cell depletion does not predict the outcome of SIV infection in monkeys[118]. In SIV-infected African monkeys, for example, bacterial translocation is prevented despite low numbers of intestinal CD4+ T cells[57]. Thus, intestinal CD4+ T-cell depletion alone cannot explain bacterial translocation and the subsequent rise in plasma lipopolysaccharide (LPS) levels in chronic HIV infection[56]. Preferential depletion of Th17 cells is associated with disruption of the intestinal barrier in pathogenic retroviral infections[140,142], but overall, the mechanism linking bacterial translocation and HIV pathogenesis is not fully understood.

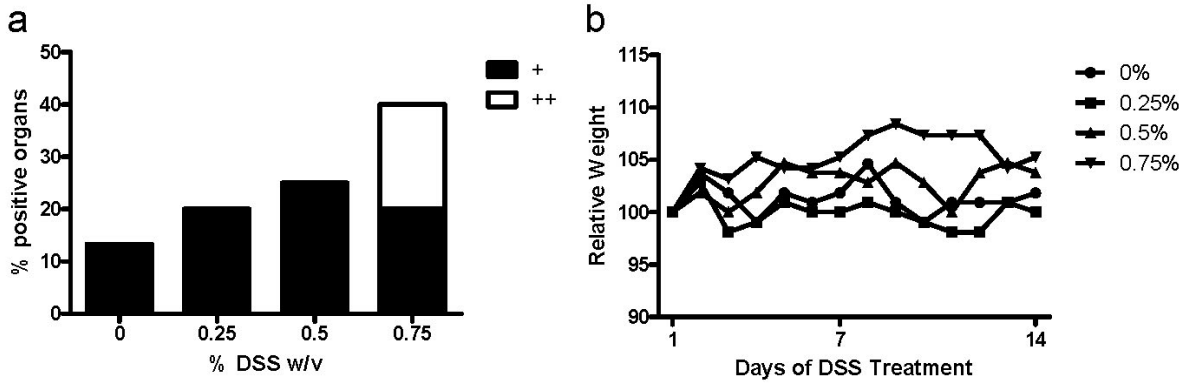
Seeking evidence for this mechanism, we examined relationships among intestinal barrier integrity, microbial translocation, immune activation, and HIV replication in a mouse-model of HIV infection. In this model, $RAG2^{-/-}\gamma_c^{-/-}$ mice are transplanted with human cord-blood hematopoietic stem cells. A human lymphoid system develops[67], and the "humanized" mice can be infected with HIV[68,79,82]. The humanized mice combine the advantages of studying HIV in human cells and in a small-animal model that facilitates experimental manipulations. Furthermore, these mice have low intestinal lymphocyte numbers[164]. In HIV infection, low numbers result mostly from virus-mediated depletion. In the humanized mice, however, they result from a low local level of engraftment by human cells. Thus, in the humanized mice, low numbers of intestinal T cells are uncoupled from the effects of viral replication. Moreover, the intestinal barrier can be disrupted by adding dextran sodium sulfate (DSS) to the drinking water of mice[87]. DSS treatment leads to apoptosis[165] and reduced proliferation of intestinal epithelial cells[166], mimicking the enterocyte apoptosis seen in SIV infection[152].

In the current study, we dissected the effects of bacterial translocation alone or in the context of HIV infection by combining DSS and HIV in humanized mice. We defined the consequences of HIV infection and bacterial translocation on plasma LPS levels, LPS clearance by macrophages, immune activation, and T-cell loss.

Results

DSS treatment or HIV infection induce a similar amount of bacterial translocation

In a DSS dose-response experiment, we established a treatment protocol that increases bacterial translocation without inducing colitis (Fig. S1). Briefly, we quantified bacterial translocation in groups of HIV-uninfected and -infected mice with mock or DSS treatment (i.e., HIV-/DSS-, HIV-/DSS+, HIV+/DSS-, or HIV+/DSS+ mice). We infected humanized mice with the CCR5-tropic HIV strain YU-2 and verified the infection by RT-PCR of plasma HIV RNA 4–6 weeks after inoculation. Thereafter, infected and uninfected control mice were treated with 0 or 0.75% DSS for 2 weeks. We cultured organ suspensions of mesenteric lymph nodes (MLN) and spleens, quantified bacterial colonies (Fig. 1A), and calculated a translocation index, based on quantity, diversity, and location of recovered bacteria (Fig. 1B). HIV-/DSS- control mice showed some baseline translocation with roughly a third of the animals containing bacteria in the MLN suspensions. Only few animals showed systemic dissemination to the spleen. DSS treatment increased bacterial translocation; in HIV-/DSS+ mice percentages of cultures containing bacteria doubled, and particularly in the spleen bacterial loads were higher.



Supplementary Figure 1: DSS induced bacterial translocation in humanized mice. 16 uninfected mice were treated with different doses of DSS in the drinking water for 2 weeks. (A) Systemic bacterial load was assessed by semi-quantitative (+ black bar, ++ white bar, +++) microbiological culture of organ suspension from mesenteric lymph nodes, spleen and liver. (B) Mouse weight as an indicator for diarrhea and colitis was measured daily (mean). This experiment was done once.

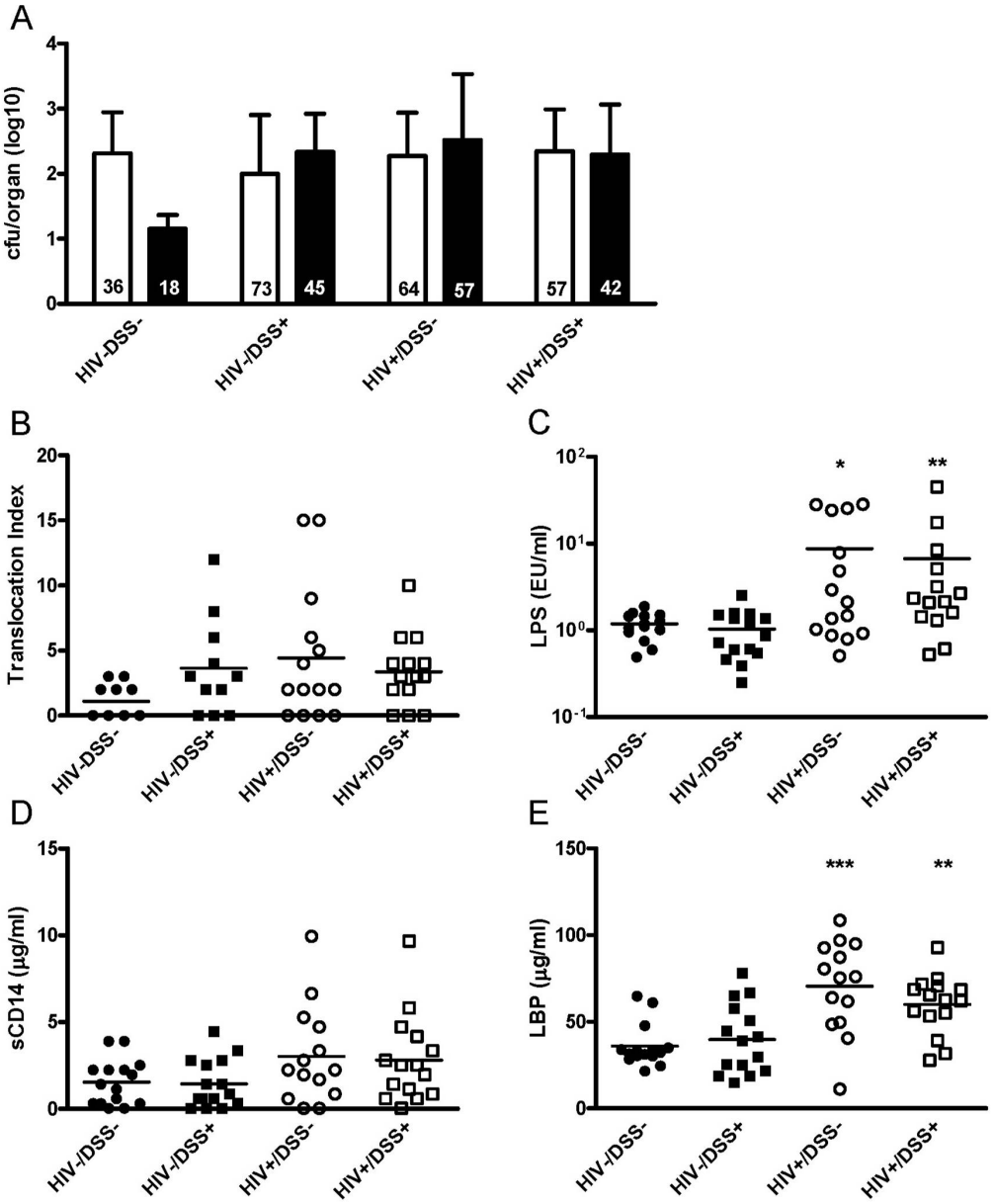


Figure 1. Gastrointestinal barrier dysfunction did not completely explain HIV-associated plasma LPS elevation in humanized mice. 4 to 6 weeks after HIV or mock infection, humanized mice received DSS 0.75% or normal drinking water for 2 weeks. (A) In cultures of mesenteric organ suspensions, bacterial colony forming units from MLN (white bar) and spleens (black bar, mean, SD) were quantified; percentages of positive organs in the different groups are indicated at the bottom of the respective bars (n=50, pooled data from two independent experiments). (B) The mice showed a trend towards higher levels of bacterial translocation (assessed by an index that includes number, species, and location of bacteria detected, P=0.1, 0.08 and 0.13, respectively) after HIV infection or DSS treatment. (C) DSS+/HIV- mice (black square) had plasma LPS levels similar to those of control mice (black circle). Only HIV+ mice without (white circle, *, P=0.015) or with DSS treatment (white square, **, P=0.005) showed significant increases of plasma LPS (n=59, pooled data from two independent experiments). (D and E) Both groups of HIV+ mice showed a trend towards higher plasma sC14 (P=0.06 for HIV+/DSS- and P=0.1 for HIV+/DSS+ mice) and had significantly higher LBP values (***, P=0.0006 for HIV+/DSS- and **, P=0.008 for HIV+/DSS+ mice).

Organ cultures yielded mainly *Lactobacilli*, *Staphylococcus xylosus*, a typical mouse commensal, and *Enterococci*, whereas stool cultures yielded a multitude of aerobic and anaerobic bacteria. Only some bacterial species translocated to the organs in sufficient numbers to be cultured. HIV+ mice had similar microbiology results to HIV-/DSS+ mice. Overall, the range and amount of translocation were comparable between HIV-/DSS+, HIV+/DSS-, and HIV+/DSS+ mice. HIV infection alone seemed to facilitate bacterial invasion from the gut, and DSS treatment did not further increase the bacterial translocation in HIV+ humanized mice.

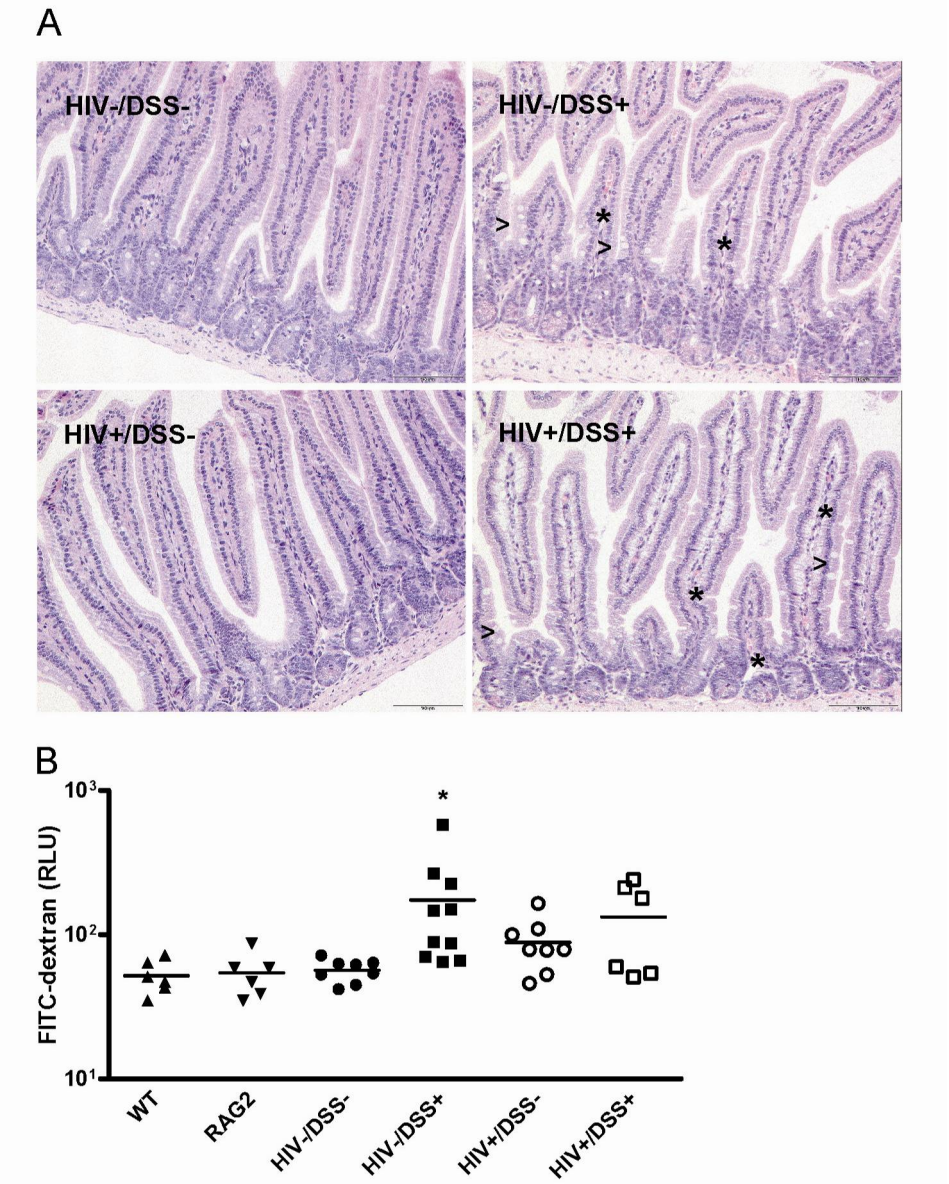
Bacterial translocation does not always induce LPS elevation

In humans bacterial translocation is quantified by measuring surrogate markers, such as plasma LPS, soluble CD14 (sCD14), or LPS binding protein (LBP). We compared these markers to the direct measurement of intestinal barrier function in humanized mice.

Plasma LPS measurements showed a contrasting picture to microbiology results. Only HIV+ mice exhibited elevated levels of LPS in the systemic circulation. HIV-/DSS+ mice, which had increased intestinal permeability according to the organ culture results, controlled plasma LPS levels (Fig. 1C). In accordance with the elevated LPS levels, HIV+ mice also exhibited higher plasma sCD14 and LBP levels (Fig. 1D and E). We did not measure endotoxin core antibodies (EndoCAb) since humanized RAG2$^{-/-}\gamma_c^{-/-}$ mice in general have very poor antibody responses, serum immunoglobulin concentrations are several log lower than in humans [67], and mostly IgM is produced with IgG appearing only several months after humanization [82].

The HIV+ mice might have had a greater influx of smaller bacterial products and as a consequence higher plasma LPS levels. To eliminate this possibility, we measured the integrity of the intestinal barrier by gavaging mice with fluorescein isothiocyanate (FITC)-dextran with a molecular weight similar to that of LPS (Fig. S2B). Furthermore, we included wild-type and non-humanized mice to determine the consequences of irradiation and transplantation. After 4 hours, FITC-dextran was detected at similar concentrations in the plasma of HIV-/DSS- control mice, wild-type BALB/c, and non-humanized RAG2$^{-/-}\gamma_c^{-/-}$ mice. Thus, independent of humanization, RAG2$^{-/-}\gamma_c^{-/-}$ and wild-type mice had equal intestinal permeabilities, and the absence of intestinal lymphocytes had little effect on permeability in this model. In DSS-treated or HIV-infected mice there was a trend towards higher FITC-dextran values. In histological sections of the intestines, we found no evidence for exacerbated damage in HIV+ mice, and DSS-treated mice showed moderate changes (Fig. S2A).

Defects in intestinal barrier integrity influenced translocation, but alone they were not sufficient to induce LPS elevation. In HIV-infected mice, some additional factors contributed to higher plasma LPS levels.



Supplementary Figure 2: Histological and functional measurement of the intestinal integrity. (A) Formalin fixed, haematoxylin and eosin stained tissue sections of HIV infected and/or DSS treated mice showed moderate changes of the intestinal mucosa. DSS treatment induced villus blunting, a modest vessel dilation (*) and discreet goblet cell hyperplasia (>). (B) Humanized mice (n=32, pooled data from two independent experiments) were infected with HIV (white symbols) or mock treated (black symbols) and 4 weeks later received 0 (circles) or 0.75% (squares) w/v DSS. After two weeks, *in vivo* permeability was measured by FITC-dextran (molecular weight 10,000, Sigma) translocation. Mice were gavaged with FITC-dextran 20 mg/20 g body weight in 200µl PBS and four hours later FITC fluorescence in the plasma was measured. Wild-type (WT, block arrow up) and non-humanized $RAG2^{-/-}c_{\gamma}^{-/-}$ (RAG2, block arrow down) mice were included as a control. HIV-/DSS+ mice had higher FITC-dextran plasma values (*, P=0.012) than WT, non-humanized and HIV-/DSS- control mice. Both HIV+ groups showed only a trend towards higher FITC-dextran translocation.

Bacterial translocation can be compensated for by increased macrophage phagocytosis

We hypothesized that plasma LPS levels are a marker for bacterial translocation and for the clearance of bacterial products from the systemic circulation. One of the main LPS clearance mechanisms is phagocytosis by liver macrophages[167,168]. We tested the influence of LPS clearance on plasma LPS levels by depleting macrophages in the humanized mice. Clodronate liposomes induce apoptosis of phagocytic cells, thus humanized mice injected with clodronate liposomes exhibited a strong reduction of macrophage numbers (Fig. 2A). When we simultaneously treated the mice with 0.75% DSS for 1 week, plasma LPS levels increased (Fig. 2B). Disturbing the intestinal barrier or reducing the number of macrophages alone caused no change in plasma LPS.

To assess macrophage function during HIV infection, we isolated liver macrophages from all four groups of mice (i.e., HIV-/DSS-, HIV-/DSS+, HIV+/DSS-, and HIV+/DSS+ mice) and incubated the cells ex vivo with FITC-LPS (Fig. 2C). All macrophages took up some LPS, but cells isolated from HIV-/DSS+ mice up-regulated their phagocytic capacity significantly compared to cells from control animals. Cells from HIV+ mice showed a slight, statistically insignificant increase of LPS phagocytosis (Fig. 2D). Further evidence of altered macrophage function in HIV+ mice was an increase of pro-inflammatory cytokines, such as IL-12 and TNF-α in the plasma (FigS3). Macrophage numbers in liver and intestines were similar in all groups (data not shown). The results imply that bacterial translocation and LPS influx can be compensated for by increased LPS phagocytosis and that this function was disturbed in HIV-infected mice.

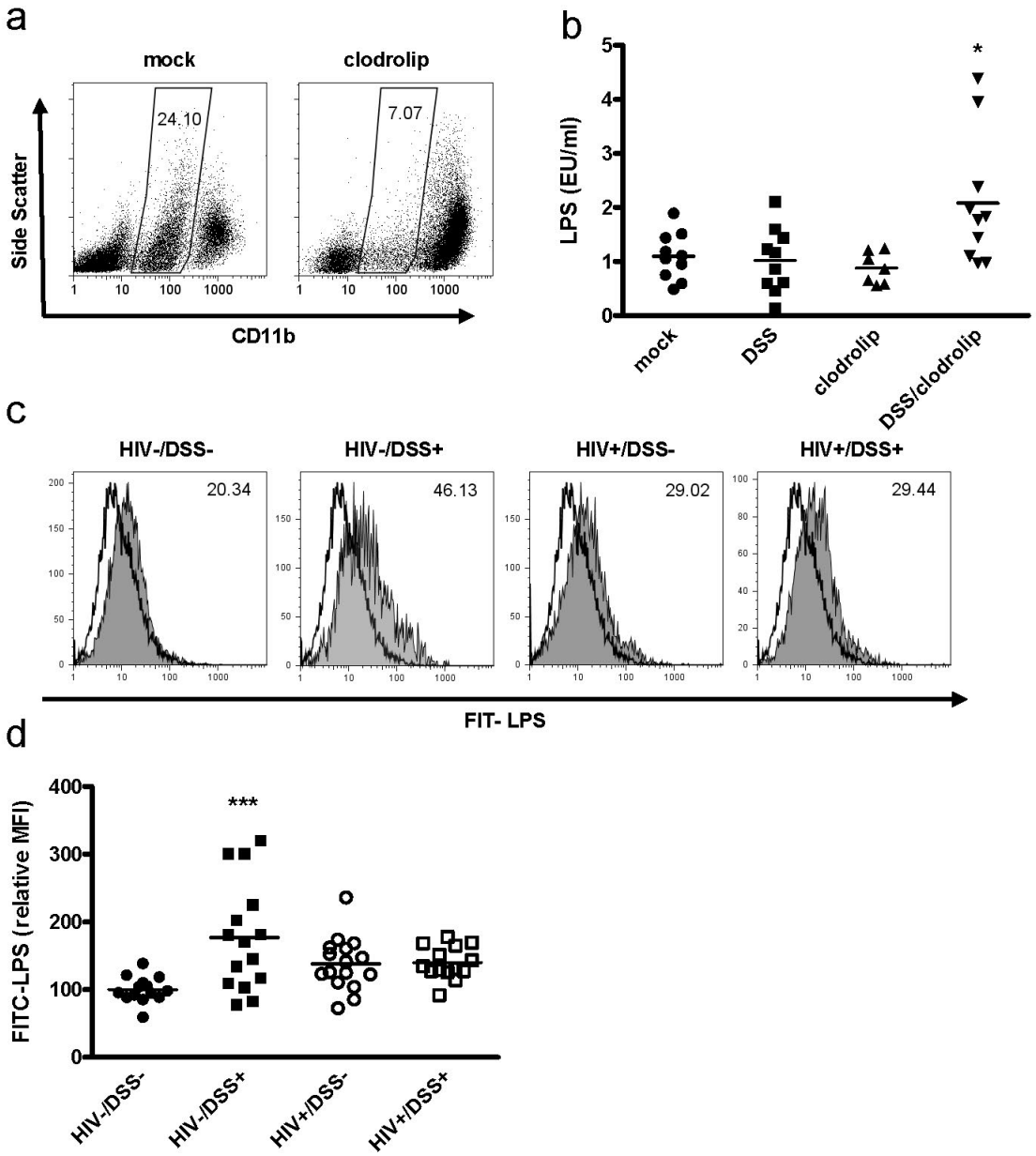


Figure 2: The combination of a bacterial translocation and disturbed LPS clearance induces plasma LPS elevation. (A) Humanized mice were injected intraperitoneally with clodrolip (1 mg/20 g body weight) to deplete phagocytic CD11b intermediate cells (spleens of representative mock PBS or clodrolip treated mice 48 h after injection). (B) After 1 week of DSS 0.75% (square, block arrow down) treatment and a second injection of clodrolip (0.5 mg/20 g body weight) (block arrow up, block arrow down), plasma LPS was only increased in mice that received both treatments (*, P=0.006, n=37, pooled data from two independent experiments). (C) Liver macrophages, isolated from HIV- or HIV+ mice that received either normal drinking water or 0.75% DSS for 2 weeks, were incubated ex vivo with FITC-LPS at 37°C or 4°C (shaded or open histogram), and mean fluorescence intensity of phagocytic cells (values upper right corner) was measured. (D) Values were normalized to the mean FITC-LPS signal of cells from HIV-/DSS- mice (black circle). DSS-induced bacterial translocation increased FITC-LPS phagocytosis (black square, ***, P<0.0001), but HIV infection abrogated this effect (white circle, P=0.2), independent of DSS treatment (white square, P=0.19) (n=59, pooled data from two independent experiments).

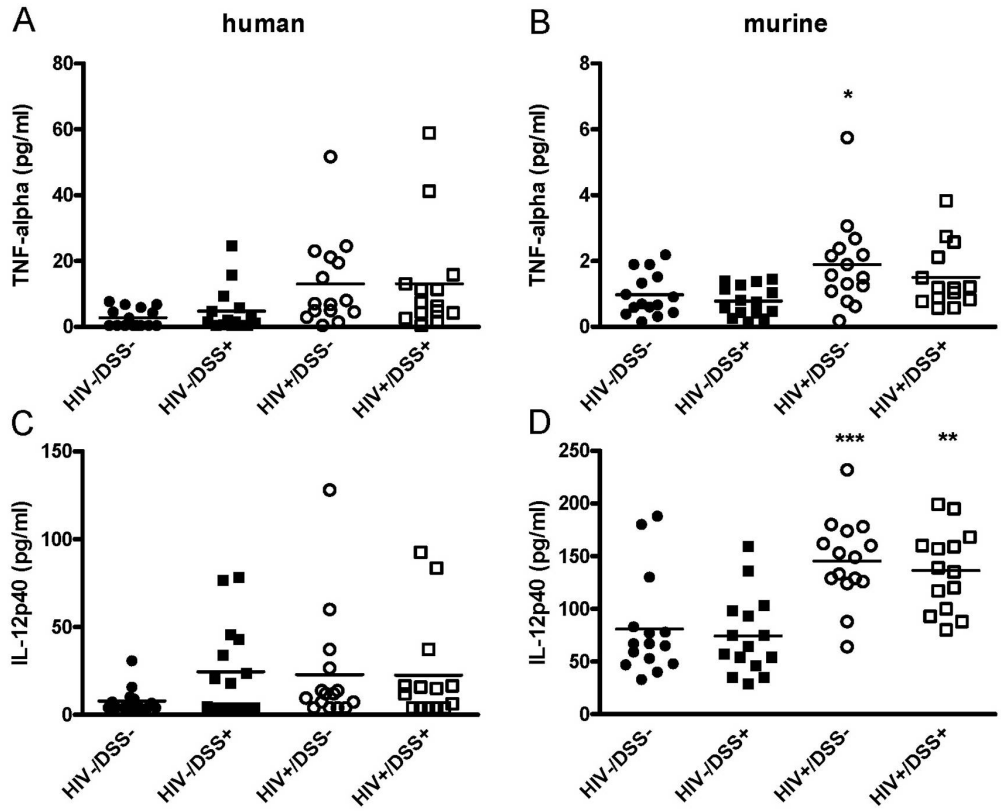


Figure S3. Macrophage derived pro-inflammatory cytokines in HIV+ mice. We measured plasma cytokine levels by cytometric bead assay (A.Urwyler, Cytolab) in HIV-/DSS- (black circle), HIV-/DSS+ (black square), HIV+/DSS- (white circle), and HIV+/DSS+ animals (white square). (A and B) Both human (left) and murine (right panel) cytokines were assessed. HIV+ mice showed a trend towards higher human (P=0.084 and 0.096) and murine TNF-alpha levels (*, P=0.045 and P=0.11 for DSS- and DSS+ mice). (C and D) Human IL-12p40 was below detection limit in many of the animals, while murine IL-12p40 was significantly elevated in HIV+ mice (***, P=0.0006, and **, 0.004 for DSS- and DSS+ mice). IL-1beta was undetectable in all mice and IL-6 values showed no significant differences (data not shown).

Bacterial translocation activates T cells

Since bacterial translocation has been implicated in immune activation during chronic HIV infection, we examined the effects of bacterial translocation alone or in the context of HIV infection on the expression of cell-surface markers of T-cell activation. We determined T-cell activation levels in the spleens of humanized mice by measuring percentages of CD38 HLA-DR double positive cells in human CD4+ or CD8+ cells (Fig. 3A). CD4+ cells from HIV-/DSS+ animals showed a slight increase of activation, but activation of CD8+ cells was more apparent (Fig. 3B). Both groups of HIV-infected mice had even higher CD8+ cell activation levels, with no difference between DSS- and DSS+ groups. There was a trend towards slightly higher CD4+ cell activation in HIV+ mice.

Thus, bacterial translocation, even if no detectable plasma LPS elevation occurred, activated CD4+ and CD8+ cells in HIV- mice. In HIV+ mice, where levels of plasma LPS were increased, CD8+ cell activation was even stronger.

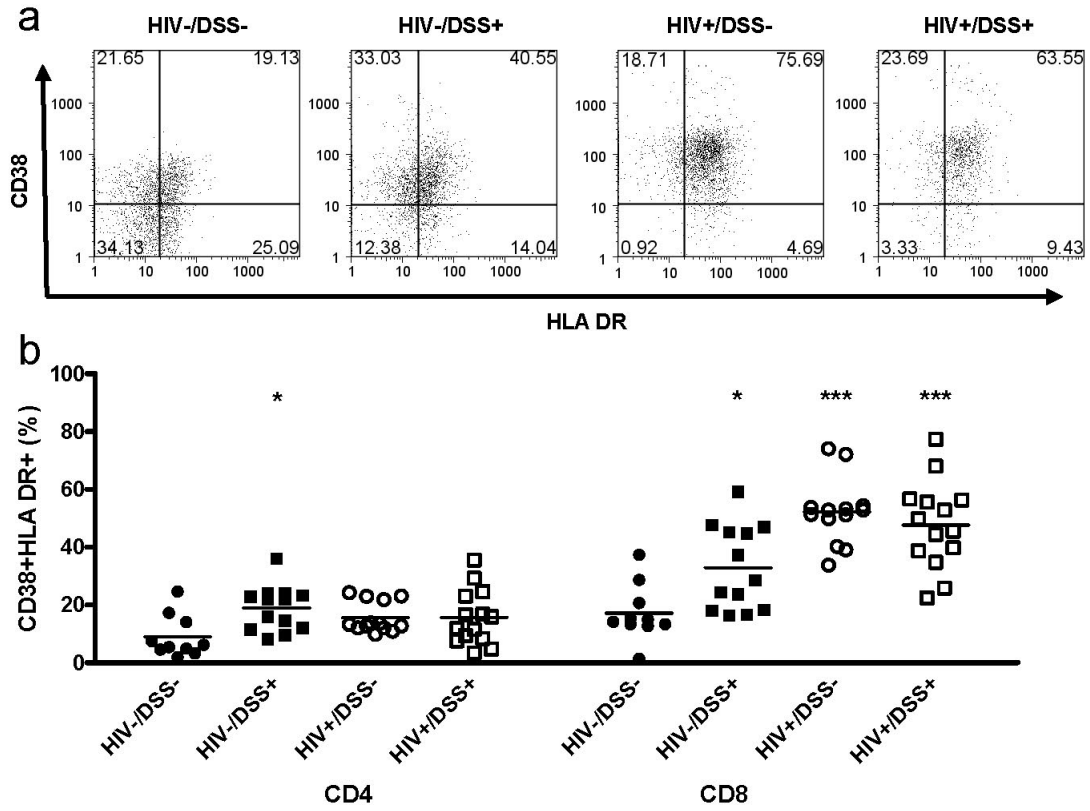


Figure 3: HIV infected humanized mice have high levels of CD8+ T cell activation. Control (black symbols) or HIV-infected mice (white symbols) received normal drinking water (circles) or 0.75% DSS (squares) for 2 weeks. (A) Activation levels in the spleen were determined by flow cytometry of human HLA-DR and CD38 staining of CD45+CD8+ (one representative animal per group) and CD45+CD4+ splenocytes. (B) DSS treatment alone slightly increased activation levels of CD4+ (*, P=0.004) and CD8+ cells (*, P=0.007), over those in uninfected, untreated control mice. HIV infection drastically increased CD8+ cell activation in animals that received normal (***, P<0.0001) or DSS (***, P<0.0001) water (n=50, pooled data from two independent experiments).

T-cell activation promotes viral replication and CD4+ T-cell loss

In HIV- mice, levels of CD4+ and CD8+ cell activation were tightly correlated (Fig. 4A). If that relationship of CD4+ and CD8+ cell activation was the same in HIV+ mice, then CD4+ cell activation levels should have been even higher in HIV+ mice than in HIV-/DSS+ mice. The correlation between activation levels of CD4+ and CD8+ cells in HIV+ mice, however, was partially lost (Fig. 4B), and the HIV+ mice had a relative deficit of activated CD4+ cells. In humanized mice, absolute CD4+ T-cell numbers differ, because the efficiency of human engraftment is variable. However, CD4+/CD8+ cell ratios are independent of engraftment and are, thus, reasonably reliable estimates of CD4+ cell depletion.

The ratios were similar for all four groups (Fig. S4A). Individual variations of CD4+/CD8+ cell ratios between mice were probably too large to detect small changes between groups in the relatively short time of the experiment. Furthermore, no difference in HIV replication was observed between HIV+/DSS- and HIV+/DSS+ mice (Fig. S4B). Nevertheless, in HIV+ mice, percentages of activated CD8+ cells correlated with lower CD4+/CD8+ cell ratios (Fig. 4C). Hence, HIV+ mice with high levels of CD8+ cell activation lost more CD4+ cells. No correlation between CD4+/CD8+ cell ratios and CD8+ cell activation was seen in HIV- mice (data not shown, $P=0.423$, $r=0.190$). In the absence of HIV infection, no loss of CD4+ cells occurred, indicating that HIV causes the preferential loss of activated CD4+ cells. Indeed, higher CD8+ cell activation levels—this time calculated in relation to total splenocytes to take engraftment level also into account—correlated with higher viral loads (Fig. 4D). In the HIV+ humanized mice, activation of CD4+ cells seemed to be masked by HIV infection and rapid loss of these cells, maybe even before full expression of activation markers.

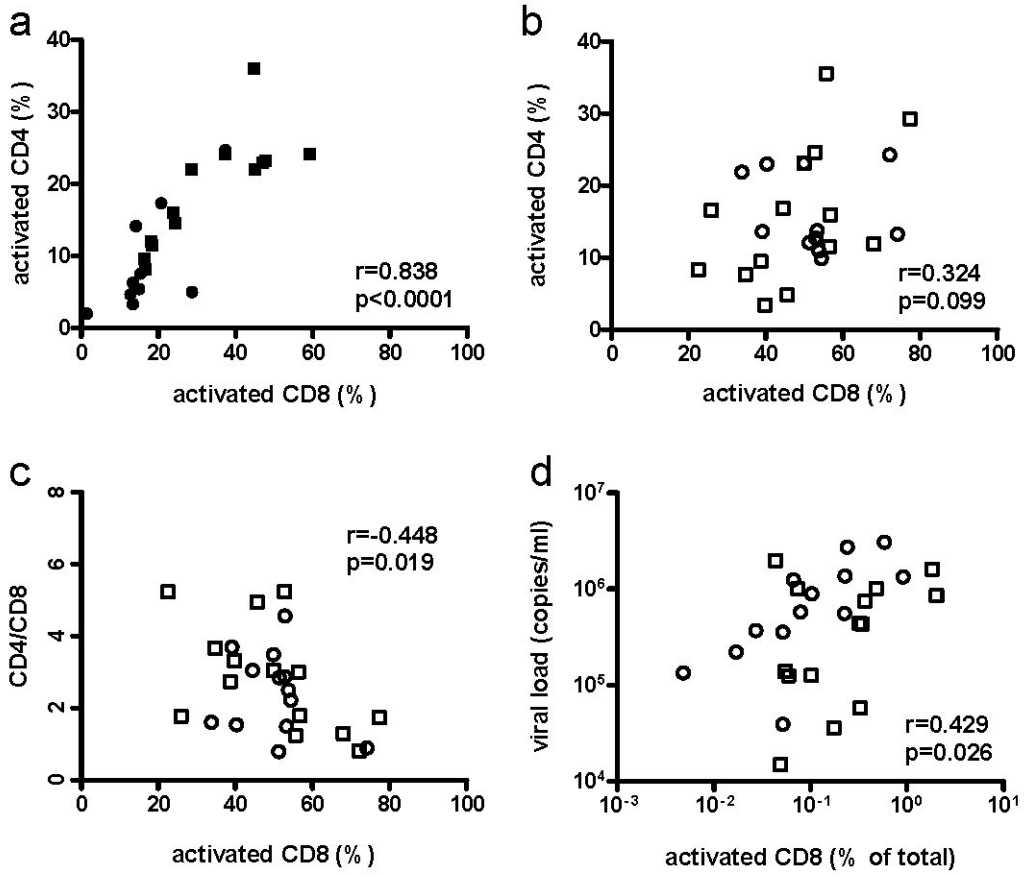
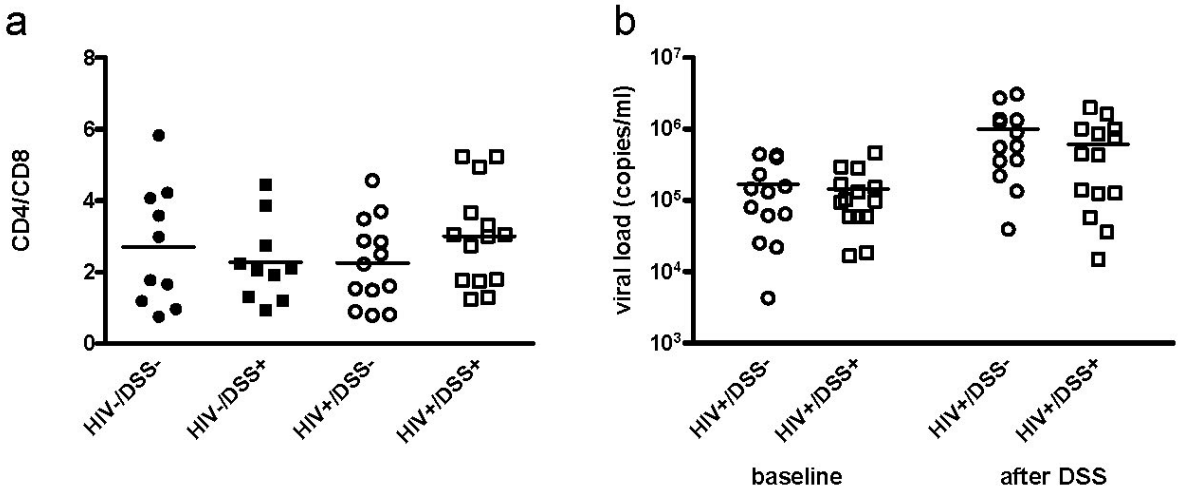


Figure 4: Activation of CD8+ cells is associated with lower CD4+/CD8+ ratios and higher viral loads in HIV infected mice. Human CD8+ splenocyte activation levels defined by HLA-DR+ and CD38+ co-staining from HIV-/DSS- (black circle), HIV-/DSS+ (black square), HIV+/DSS- (white circle), and HIV+/DSS+ animals (white square) were correlated with CD4+ cell-activation levels, CD4+/CD8+ cell ratio, and viral load. (A) In uninfected mice, activation levels of CD8+ and CD4+ cells were tightly correlated (n=23, pooled data from two independent experiments). (B) But in HIV+ mice, this relationship was not as clear (n=27, pooled data from two independent experiments). (C) In HIV+ mice, higher percentages of activated CD8+ cells correlated with lower CD4+/CD8+ cell ratios. (D) When activation levels were adjusted for overall engraftment by calculating percentages of HLA-DR+CD38+CD8+ cells of total cells, including murine cells, CD8+ cell activation correlated also with higher viral loads.



Supplementary Figure 4: Diversity of CD4/CD8 ratios and viral loads in humanized mice. Humanized mice were infected with HIV (white symbols) or mock treated (black symbols) and 4 weeks later received 0 (circles) or 0.75% (squares) w/v DSS. (A) After 2 weeks, spleens were removed and splenocytes were analyzed for human CD4+ and CD8+ T-cell ratios by flow cytometry (n=50, pooled data from two independent experiments, no significant differences). (B) Plasma viral load was measured (n=27, pooled data from two independent experiments), 48 h before the beginning of DSS treatment (baseline, no significant difference), and at the end of the experiment (after DSS, no significant difference).

Discussion

Dysfunction of the intestinal barrier has severe consequences for the whole organism. It leads to translocation of bacteria from the gut and mediates inflammation. In chronic HIV infection, for instance, elevated levels of circulating bacterial products are associated with T-cell activation and disease progression[56]. We established a humanized mouse model of intestinal barrier dysfunction to determine the systemic effects of bacterial translocation. In our model, permeability corresponded well with translocation. But plasma LPS levels, the classical marker of bacterial translocation, depended only partially on barrier dysfunction. Macrophages compensated for the increased bacterial translocation by up-regulating their phagocytic capacity and thereby kept plasma LPS levels in a normal range. In HIV-infected mice, however, LPS clearance was inadequate leading to increased plasma LPS levels, high T-cell activation, and vigorous HIV replication.

A multilayered barrier protects the body from invading intestinal bacteria. The first line of defense is a tight, mucus-coated sheath of intestinal epithelial cells. Moreover, leukocytes in the underlying lamina propria contribute to the protection against bacteria. The humanized mice we used have low intestinal lymphocytes numbers[164]. Therefore, changes of the epithelial integrity probably have a relatively big impact on translocation in humanized mice, even without obvious histopathological changes. While there was no obvious difference in FITC-dextran translocation between humanized and wild-type mice, intestinal permeability tended to increase after HIV infection or low dose DSS treatment (Fig. S2B). Notably, HIV+ mice had high plasma TNF-α levels (Fig. S3). TNF-α disrupts tight junctions and induces apoptosis of intestinal epithelial cells[169], thereby mediating barrier dysfunction. Baseline translocation in HIV-/DSS- mice was quite high with one third of the MLN cultures containing bacteria (Fig. 1A and B). HIV+ or DSS+ mice more frequently had bacteria translocating to MLN and spleen—in accordance with the increased FITC-dextran permeability. From our data, it is not possible to infer the exact role disturbance of epithelial permeability and intestinal lymphocyte depletion plays in human HIV infection. But in general, the amount of bacterial translocation depends on barrier integrity.

Surprisingly, plasma LPS levels showed a different pattern (Fig. 1C). They did not depend strictly on permeability and translocation. In our experiments, HIV+ mice had elevated plasma LPS levels. HIV-/DSS+ mice controlled the increased influx of bacteria from the gut by raising their ability to clear LPS. LPS elevation only occurred in animals that had

disturbance of the intestinal barrier and at the same time LPS clearance defects, either because of macrophage depletion or HIV infection (Fig. 2). Thus, macrophage phagocytosis seems to be critical in protecting against systemic translocation and failed clearance leads to systemic elevation of bacterial products. This hypothesis is supported by studies of inflammatory bowel disease. Plasma LPS levels are elevated in active inflammatory bowel disease, when the intestinal barrier is disrupted[123]. Furthermore, patients with Crohn's disease clear subcutaneously injected bacteria very slowly, their macrophages secrete few pro-inflammatory cytokines in response to bacteria or TLR ligands, and the transcription profiles of these macrophages show defects in vesicle trafficking and cytoskeletal organization [170]. This indicates that defects similar to the dysfunctional macrophage phagocytosis seen in our model might be important in inflammatory bowel diseases.

The mechanism leading to macrophage dysfunction in HIV+ mice is not clear. It is tempting to postulate induction of an "endotoxin-tolerant" macrophage phenotype in HIV-/DSS+ mice, and loss of tolerance induction in HIV+ mice. Endotoxin tolerance is characterized by programming of macrophages towards phagocytosis instead of production of pro-inflammatory cytokines upon LPS re-exposure[171]. Indeed, plasma IL-12 and TNF-α levels were normal in HIV-/DSS+ mice despite increased bacterial translocation. In contrast, HIV+ mice had high levels of pro-inflammatory cytokines (Fig. S3). At the moment, the factors inhibiting tolerance induction are unknown. Duration of translocation might play a role: HIV-/DSS+ mice had barrier dysfunction for the relatively short period of two weeks. Control of longer lasting translocation might not be as easy. Otherwise, the pro-inflammatory state generated by HIV infection might influence macrophage function. Macrophages integrate a broad range of environmental information. Some cytokines[172] and bacterial products[173,174] sensitize cells to LPS stimulation. Furthermore, viral products and/or cytokines produced due to virus infection interfere with endotoxin tolerance induction in chronically HCV-[175] or HIV-[176] infected patients. Instead monocytes from these patients produce more TNF-α upon LPS re-stimulation.

So far, it is unknown if LPS clearance dysfunction also exists in HIV-positive humans, although some evidence supports the existence of macrophage defects in chronic HIV infection. Plasma LPS levels in acutely HIV-infected patients are not elevated[56], despite early depletion of gut lymphocytes. During treatment interruption, LPS levels rise only after a few weeks of viral replication[124]. Early after antiretroviral therapy is stopped, falling EndoCAb levels indicate functional LPS clearance. Thereafter, elevation of LPS levels suggests the

onset of clearance defects. In fact, HIV inhibits macrophage maturation[177] and phagocytosis[178,179]. Monocytes from HIV-infected individuals show impaired *Mycobacterium* phagocytosis[180]; *Saccharomyces* up-take is also decreased, and LPS-mediated enhancement of phagocytosis is less than the enhancement in monocytes from healthy donors[181]. Moreover, monocytes from HIV-infected patients show an attenuated pro-inflammatory cytokine response to LPS stimulation ex vivo[56,182], maybe due to *in vivo* pre-stimulation. Serum IL-12 and TNF-α levels, which reflect *in vivo* cytokine production, are higher[183-186]- similar to the increased cytokine levels we observed in HIV+ mice. Overall, our findings in HIV-infected humanized mice resemble the results from HIV-infected humans.

Certainly, not all aspects of human HIV infection can be modeled accurately in humanized mice. For example, direct HIV infection of macrophages is rare in the mice. Engraftment of human monocytes was low; less than 2% of all monocytes were of human origin (data not shown). Most macrophages are of murine origin and therefore resistant to HIV infection. In humans, macrophage permissiveness to HIV infection varies from tissue to tissue. For example, intestinal macrophages are quite resistant to infection, but vaginal macrophages are readily infected[187]. However, productive infection of macrophages is, in general, infrequent *in vivo*[70], except for late stages of disease when opportunistic infections occur[188]. To definitely determine the importance of direct viral infection of macrophages, other models with bigger populations of human myeloid cells would be useful.

Lymphoid engraftment, however, is quite good in humanized mice. This allowed the investigation of T-cell activation in DSS-treated or HIV-infected mice. Bacterial translocation induced CD4+ and CD8+ cell activation (Fig. 4). Other activating factors (i.e., stimulation of TLR7/8 by HIV RNA[189-191]) might also have played a role. However, in HIV-/DSS+ mice, such other activating factors were not present, and bacterial translocation alone activated T cells. Since plasma LPS levels were not elevated in these mice, immune activation might have been mediated by other bacterial components, such as peptioglycan, flagellin, or bacterial DNA. Moreover, LPS flux—input from the gut and subsequent clearance—was greater in these mice. A greater LPS flux might activate the immune system. At least, it induced and sustained stimulation of macrophage phagocytosis. Uncontrolled bacterial translocation, as seen in HIV+ mice, might reasonably caused higher levels of lymphocyte activation. Not surprisingly, DSS treatment had no effect on T-cell activation in HIV+ mice. The bacterial translocation indices and LPS phagocytosis capacities were almost identical in HIV+/DSS-

and HIV+/DSS+ groups, leading to similar LPS, LBP and sC14 levels and, therefore, to similar activation levels.

Our results might explain why levels of CD8+ T-cell activation are especially good markers of disease outcome[72], even though CD8+ T cells are not direct viral targets. In HIV-uninfected mice CD8+ and CD4+ cell activation levels were tightly correlated. CD8+ T-cell activation levels seem to predict the amount of activated and HIV-permissive CD4+ T cells. These cells have a very short lifespan; the half-life of an infected CD4+ T cell is estimated at 1.6 days[192]. Additionally, measurements of lymphocyte telomere lengths indicate that, in HIV-infected individuals, only CD8+ T-cell turnover is increased, while telomeres in CD4+ T cells show no excessive turnover[193]. CD4+ T cells might be lost due to HIV infection before cell division is accomplished. This could also explain the relative deficit of activated CD4+ cells we observed in HIV+ animals. HIV preferably infects activated CD4+ cells[144]. Indeed, those mice with the highest percentages of activated CD8+ cells had the lowest CD4+/CD8+ cell ratios and the highest HIV plasma loads.

In conclusion, we identified a critical role of macrophages in protection from systemic bacterial translocation. In humanized mice, failure of LPS clearance was associated with high levels of T-cell activation and HIV replication. Macrophage dysfunction might be an underestimated mechanism in HIV-induced immunodeficiency and certainly warrants further investigation.

Material and Methods

Ethics Statement

All experiments were approved by ethical committees of the University Zurich and the Federal Veterinary Department and were conducted according to local guidelines (TschV, Zurich) and the Swiss animal protection law (TschG). Cord blood was collected with written consent of the parents.

Generation and HIV infection of humanized mice

Mice were reconstituted and infected as described[68]. Briefly, newborn RAG2$^{-/-}$c$_\gamma^{-/-}$ mice were irradiated with 2x2 Gy. CD34+ cells were isolated from human cord blood with immunomagnetic beads (Miltenyi Biotec), and 2.75 ± 0.5 x 10^5 cells were transplanted into each mouse. After 10 to 16 weeks, the degree of blood engraftment was determined by flow cytometry of peripheral blood mononuclear cells stained for the panhuman marker CD45 in all mice (mean human cells/live cells 5.6 ± 5.4% SD). Mice were infected intraperitoneally with HIV YU-2, 2 x 10^5 of the tissue-culture infectious dose$_{50}$ per mouse. Plasma viral loads were measured by RT-PCR (Amplicor, Roche) 4–6 weeks after inoculation and at the end of each experiment. The detection limit was 1,600 HIV RNA copies/ml. Activation levels of T cells were measured by flow cytometry of splenocytes stained for human CD45, CD4, CD8, HLA-DR, CD38, and appropriate isotype controls (all from BD Biosciences). In all experiments, mouse litters and cord blood donors were evenly distributed to all experimental groups.

Induction and measurement of bacterial translocation

Bacterial translocation was induced by adding 0.75% (w/v) DSS (molecular weight 40,000, MP Biomedicals) to the drinking water for 2 weeks. Spleen and MLN were removed aseptically, mashed with a pestle in PBS, and plated on sheep blood and MacConkey agar plates. Plates were only incubated aerobically, since in a pilot experiment, no anaerobic bacteria could be detected in organ cultures. As a control, diluted stool samples were cultured both in aerobic and anaerobic conditions. A descriptive bacterial translocation index was calculated from organ culture results (no bacterial growth = 0 points, 10 to 99 cfu/organ = 1 point, 100 to 999 cfu/organ = 2 points, >1000 cfu/organ = 3 points, for all organs points were multiplied by the number of bacterial species detected. For spleen cultures, points were

doubled, and finally, all points from one mouse were added up). LPS was quantified by endpoint chromogenic limulus amoebocyte lysate assay (Lonza). Plasma samples were diluted 1:10 with endotoxin free water, incubated at 85°C for 12 min, and assayed according to the manufacturer's instructions. Standard endotoxin (Lonza) was diluted to cover plasma LPS values within a range of 0.5 to 20 EU/ml. Plasma mouse sCD14 and LBP were measured by ELISA (both from CellSciences), according to the manufacturer's instructions. For sCD14 measurement, samples were diluted 1:150, and for LBP measurement, 1:800.

Ex vivo LPS phagocytosis

Liver mononuclear cells were isolated by Ficoll-Hypaque density gradient centrifugation. To further purify macrophages, cells were incubated for 3 h in RPMI, 10% FCS, penicillin/streptomycin, and L-glutamine at 37°C, 5% CO_2, and then washed two times with room temperature PBS to remove non-adherent cells. This procedure yielded over 90% murine CD11b+ cells. Cells were then incubated with 0.1 mg/ml FITC-LPS (Sigma) in RPMI, 10% FCS, penicillin/streptomycin, and L-glutamine at 37°C, 5% CO_2 for 1 h. As a control, cells were also incubated with FITC-LPS at 4°C. Cells were then washed two times with cold PBS, detached with trypsin, washed once again, and analyzed by flow cytometry. FITC-LPS mean fluorescent intensity was normalized to the fluorescence of samples from HIV-/DSS- mice.

Macrophage depletion

Mice were injected intraperitoneally with clodrolip (1 mg/20 g body weight) or with PBS[194]. After 48 h, depletion was verified in four mice by staining of spleen and liver cells for CD11b. To maintain depletion, mice were treated a second time with clodrolip 0.5 mg/20 g body weight or with PBS intraperitoneally 4 days after the first injection. Concurrently, half of the mice received DSS 0.75% (w/v) in their drinking water. After 1 week, the mice were sacrificed, and their plasma LPS levels were measured.

Statistical analysis

GraphPad Prism 5.02 was used for statistical analysis. Data were analyzed by parametric one-way analysis of variance, followed by Bonferroni post-test. All p-values shown are adjusted for multiple comparisons. In the figures, p-values are presented for comparisons between treatment groups and controls and are denoted by asterisks. Values for HIV/DSS experiments

for plasma LPS, viral load, FITC-dextran fluorescence, and percentages of activated CD8+ cells (of total cells) were log transformed before analysis to reduce right-skewing of the data. For all correlations, Pearson's correlation coefficient was calculated. In all figures, points represent values of individual mice, and lines depict mean values.

Acknowledgements

We thank M. Ito and M. Heikenwalder for providing mice, the staffs of the Microbiology Laboratory at the Children's University Hospital Zurich, of the Maternité at the Triemli Hospital Zurich, of the Clinical Immunology Laboratory at the University Hospital Zurich, and of the Animal Facilities at the University Zurich for their support. We thank Miranda Smith for technical advice, Adrian Urwyler for measuring cytokines, and Roche for providing RT-PCR reagents. UH is supported by the Swiss National Science Foundation (SNSF) with a scholarship (323530-123717) and by a grant (1291) from the Hartmann Müller Foundation, Zurich. RFS is supported by the SNSF (1003A-118391). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. The authors have no competing financial interests.

Contribution

UH initiated and designed the project, conducted and analyzed all experiments, and wrote the manuscript. ES, SB, and MN assisted in some experiments. RS provided liposomes and assisted in the design of macrophage depletion experiments. SR was responsible for HIV RNA PCR. DN was responsible for microbiological analysis of organs. WK performed immunohistochemistry. RFS supervised the project.

5. Conclusions and Outlook

5.1 Mouse models of rectal HIV transmission

Intestinal engraftment and transmission rates in different models

Millions of people worldwide are infected with HIV. Despite improved treatment strategies and efforts in prevention, no end of the pandemic is in view[195]. A major goal is to prevent new HIV infections[74]. For a rationale design of prevention methods, we need a detailed knowledge of HIV transmission and the early steps in HIV infection[196]. However, the field of HIV research has long been hampered by the lack of a small animal model that mirrors HIV infection in humans[62] and permits to study questions such as vaginal or rectal transmission[61].

Recent advances in the generation of humanized mice now allow experiments with HIV *in vivo*. Injection of human cord blood $CD34^+$ cells into newborn $RAG2^{-/-}\gamma_c^{-/-}$ mice leads to development of human T, B, and dendritic cells, and successive formation of primary and secondary lymphoid organs[67], and in these humanized mice experiments with HIV can be performed *in vivo*[68]. In the current work, we evaluated this model for studying rectal HIV transmission. We found that in the humanized intestinal engraftment was disproportionately low compared to engraftment in other lymphoid organs. Thus, rectal HIV transmission rarely was successful, even in conditions associated with a high transmission risk. Neither local treatment with the pro-inflammatory cytokine IL-1β nor colitis induction with DSS increased rectal HIV transmission rates (**Chapter 2**). Additionally, we explored the potential of seminal components, known to enhance HIV infection rates *in vitro*[90], to facilitate rectal HIV transmission in our humanized mouse model, but no transmission enhancing effect was detectable *in vivo* (data not shown). Therefore, the value of humanized $RAG2^{-/-}\gamma_c^{-/-}$ mice to investigate rectal HIV transmission is limited.

Currently, mice transplanted with human fetal organs, so called BLT mice, are the only mice allowing reliable HIV transmission via the rectal route[66]. Furthermore, they are useful to study new prevention strategies[197]. BLT mice have good intestinal engraftment with human T, B, and myeloid cells. Furthermore, the majority of human T-cells in the gut of BLT mice show a memory phenotype similar to the one observed in lymphocytes isolated form the human intestinal mucosa[66]. Thus, the GALT of BLT mice resembles the human GALT in many aspects. At the moment, the BLT mouse model seems superior to humanized $RAG2^{-/-}\gamma_c^{-/-}$ mice for studying rectal HIV transmission.

While co-transplantation of fetal organs and hematopoietic stem cells facilitates and enhances engraftment of human cells and might thereby induce repopulation of the GALT, it is rather

laborious. BLT mice are generated by first surgically implanting pieces of human fetal liver and thymus, and then, after a healing period, the mice are sublethally irradiated and transplanted with relatively big number of corresponding stem cells. Consequently, ethical and practical reasons prohibit the large-scale use of BLT mice.

Future directions

Modified humanized mouse models might provide valuable alternatives to BLT mice for studying mucosal HIV transmission. Human cord blood derived hematopoietic stem cells are widely available and straight forward to isolate, and therefore are the obvious choice as transplant source. As transplant recipients a wide range of different mouse strains exist. Other immunodeficient mice despite $RAG2^{-/-}\gamma_c^{-/-}$ mice might favour human intestinal engraftment. NOD/SCID $\gamma_c^{-/-}$ (NOG) mice, for instance, have a higher overall, and in particular T-cell engraftment than $RAG2^{-/-}\gamma_c^{-/-}$ mice[198]. In preliminary experiments, humanized NOG mice seemed to have some human GALT engraftment, although skewed towards the B-cell lineage (data not shown). Moreover, the transgenic expression of human factors, such as human MHC or human cytokines, might further improve level and range of human immune system development in humanized mice.

5.2 Bacterial translocation as a cause of HIV pathogenesis

Mechanistic model

Chronic immune activation is a distinct feature of HIV infection[199]. It is one of the best prognostic markers for disease progression[200], it is associated with impaired immune reconstitution in patients on antiretroviral therapy[201], and it distinguishes pathogenic from non-pathogenic simian immunodeficiency virus (SIV) infection in monkeys[59,202]. It is characterized by an increase in T-cell turnover[203], in B-cell dysfuntion[204], in frequencies of T-cells with an activated phenotype[205], and in serum levels of pro-inflammatory cytokines[206]. The immune system is put under a constant strain leading to dysregulation and exhaustion of immune functions, and finally immunodeficiency[207]. However, the underlying causes of immune activation in chronic HIV infection are poorly defined (reviewed in **Chapter 3**).

A potential mechanism is immune activation by circulation of microbial products from the gut. Previous studies showed that circulating lipopolysaccharide (LPS), a marker of microbial translocation from the gastrointestinal tract, is increased in chronically HIV infected individuals and correlates with immune activation levels[56]. With our unique model of DSS treated and/or HIV infected humanized mice, we established an *in vivo* model of bacterial translocation and HIV pathogenesis (**Chapter 4**). Using this model we identified a new facet of HIV induced immunodeficiency, namely inappropriate LPS phagocytosis by macrophages. This defect initiates a cascade of uncontrolled bacterial translocation, LPS elevation, T-cell activation, and HIV replication (Figure 1). These results propose new ways of thinking about the role of bacterial translocation in chronic HIV infection; they emphasize the importance of macrophages in controlling the systemic load of bacterial products. Restoring appropriate clearance of translocated bacteria might be beneficial in preventing some of the negative effects of chronic HIV infection.

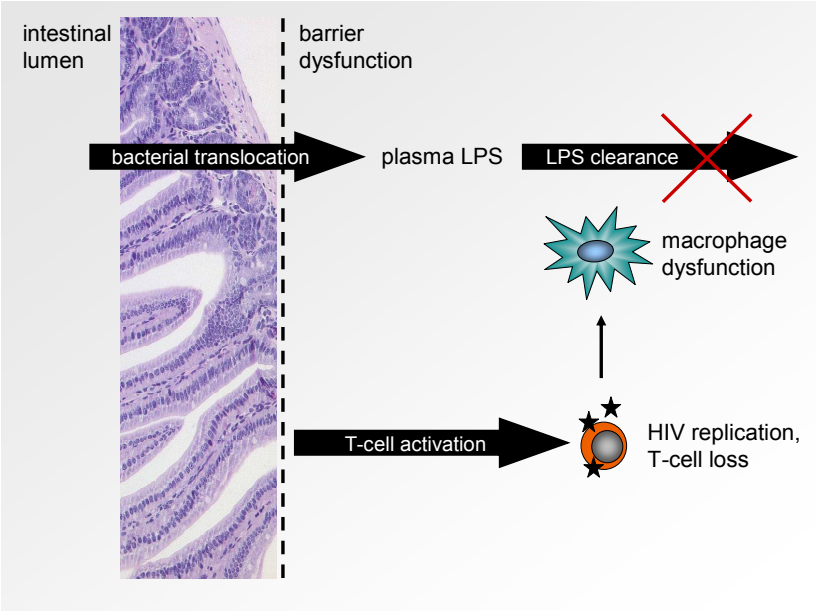


Figure 1. Uncontrolled bacterial translocation fuels HIV pathogenesis. HIV infection leads to an increased intestinal permeability and bacterial translocation from the gut. Additionally, macrophages are not able to clear the incoming LPS, leading to a vicious circle of increased T-cell activation and viral replication.

Future directions

Validation of our results with samples from SIV infected monkeys and HIV infected humans is a priority. Some evidence suggests the presence of similar macrophage defects in these situations (discussed in **Chapter 4**), but data are incomplete. General phagocytosis defects have been documented, but LPS phagocytosis has not been studied so far. Optimally, primary liver macrophages should be used for such studies. To get such samples from HIV infected humans is however quite difficult. Therefore, mouse models with improved human myeloid engraftment are a valid alternative as a source of HIV-infected tissue macrophages. In our current RAG2$^{-/-}\gamma_c^{-/-}$ mouse model, most myeloid cells are of murine origin, and thus, HIV resistant. Therefore, we assume that the macrophage dysfunction we observed in the current study is mainly an indirect consequence of HIV infection. The same indirect effect of HIV on macrophages might be important in humans. Productive HIV infection of macrophages in humans is rare[70].

In a next step, elucidation of the factors regulating macrophage function is essential. Viral products might directly affect macrophage phenotype, this could include TLR7 triggering by HIV ssRNA[68] or Tat-mediated changes[208]. Indirect effects might include phagocytosis of infected, apoptotic cells or cytokines produced by infected cells that activate macrophages. Furthermore, translocated, bacterial PAMPs probably will determine functional properties of the macrophages. *In vitro* experiments with myeloid cell lines or monocyte derived macrophages will be helpful in deciphering the influence of these different factors on macrophage phenotype and function.

6. Abbreviations

AICD	activation induced cell death
AIDS	acquired immunodeficiency syndrome
BLT	bone marrow liver thymus
ca	cell-free
cf	cell-associated
CFSE	carboxy fluorescein succinimidyl ester
cfu	colony forming units
clodrolip	clodronate liposomes
CTLA	cytotoxic T lymphocyte antigen
DAPI	diamidino-phenylindol
DC	dendritic cell
DNA	desoxyribonucleic acid
DSS	dextran sodium sulphate
ELISA	enzyme linked immunosorbent assay
EndoCAb	endotoxin core antibody
EU	endotoxin unit
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FITC	fluoresceinisothyocyanate
GALT	gut associated lymphoid tissue
gp	glycoprotein
Gy	gray
HAART	highly active antiretroviral therapy
H&E	hematoxylin and eosin
HIV	human immunodeficiency virus
IEL	intraepithelial lymphocyte
IL	interleukin
IFN	interferon
LBP	LPS binding protein
LI	large intestine
LPL	lamina propria lymphocyte
LPS	lipopolysaccharide
MACS	magnetic cell separation
MFI	mean fluorescence intensity

MHC	major histocompatibility complex
MLN	mesenteric lymph node
NK cell	natural killer cell
NOD	non obese diabetic
NOG	NOD/SCID $\gamma_c^{-/-}$
PBL	peripheral blood leukocytes
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PHA	phytohemagglutinin
RAG	recombinase activating gene
RLU	relative light units
RNA	ribonucleic acid
RT-PCR	reverse transcription-polymerase chain reaction
sCD14	soluble CD14
SCID	severe combined immunodeficiency
SD	standard deviation
SI	small intestine
SIV	simian immunodeficiency virus
SP	seminal plasma
SSC	side scatter
STAT	signal transducer and activator
$TCID_{50}$	tissue culture infectious dose 50
thy/li	thymus/liver
TLR	toll like receptor
TNF	tumor necrosis factor
Treg	T regulatory cell
WT	wild type
γ_c	common gamma chain

7. Acknowledgments

I am thankful for the support of all the members of the Speck Lab. First and foremost, I greatly appreciated the opportunity to do exciting research. Professor Speck trusted me to pursue own ideas and projects and gave me a lot of freedom. Without the help of all my colleagues in the lab this thesis wouldn't have been possible. I always could count on them when I had to deal with technical difficulties, large experiments, demanding reviewers, or when I hit a scientific dead end.

I'd like to thank the members of my thesis committee. They always gave great scientific input and several key experiments are based on their suggestions. I'm particularly grateful to Professor Brunner for technical advice and for inviting us to write a review.

Many thanks go to the Zurich MD-PhD committee, especially to the Professors Aguzzi and Trkola. The Zurich MD-PhD program helped me tremendously to find my way into science. The program enabled me to get a SNF fellowship and provided me with a thorough education not only of scientific knowledge but also of how to be a scientist. Furthermore, I appreciated the support and friendship of my MD-PhD peers, their enthusiasm and curiosity always gave me a boost of new motivation.

I am thankful for the support of the Infectiology Department at the University Hospital Zurich. Professor Weber provided not only the opportunity to conduct research but also to present the results at international conferences. All members of the HIV lab were great colleagues and friends.

Furthermore, I greatly appreciate the financial support I received from the Swiss National Science Foundation and the Hartmann Müller Stiftung. This personal funding motivated me to get results and to publish them.

Special thanks go to my partner, family and friends who stood by me during my MD-PhD years and reminded me that there is a life outside of the lab.

8. Curriculum Vitae

Ursula Hofer

Address	University Hospital Zurich	Date of Birth:	12.06.1980
	Division of Infectious Diseases	Nationality:	Swiss, from
	and Hospital Epidemiology		Schüpfen, Bern
	Raemistrasse 100	E-mail:	hoferu@gmail.com
	CH-8091 Zurich		

Education

Since 2007	PhD studies in immunity and infection biology
	MD-PhD program, University and ETH Zurich, Switzerland
	Thesis: *HIV's interactions with the intestinal mucosa*
	Supervisor: Professor Roberto Speck
2004-2006	MD Thesis, University of Bern, Switzerland
	Thesis: *Non-phagocytic entry of nanoparticles in a co-culture model of the lung*
	Supervisor: Dr. Sc. nat. ETH Barbara Rothen-Rutishauser
2000-2006	Medical school, University of Bern, Switzerland
2000	Matura, Typus B, Biel-Bienne, Switzerland

Honors and Awards

2010	Conference scholarship
	Keystone Symposia, HIV Pathogenesis
2009	Research grant
	What is the impact of bacterial translocation and immune activation on HIV pathogenesis in vivo?
	Hartmann Müller Foundation, Switzerland

2008	MD-PhD fellowship
	Swiss National Science Foundation
2006	Award for the best Federal Exam of the Medical Faculty
	University of Bern, Switzerland

Research Experience

PhD	*In vitro* and *in vivo* studies of HIV infection in the gut associated lymphatic tissue and of the systemic consequences of HIV induced bacterial translocation
	Immunomodulatory therapies in various mouse models of viral infection (HIV in humanized mice, LCMV, Friend Retrovirus)
MD	Development of an *in vitro* system to study nanoparticle and influenza virosome up-take in pulmonary epithelial and primary immune cells

Related Professional Experience

Since 2008	Resident physician, 20%
	Division of Clinical Immunology
	University Hospital Zurich, Switzerland
2007	Resident physician
	Department of Internal Medicine
	Regional Hospital Einsiedeln, Switzerland
2006	Medical advisor and writer for a website providing patient information
	www.eesom.com

Teaching

2007-2009	Teaching assistant, University Zurich
	Medical Immunology course
2006	Teaching assistant, University of Bern
	Fluorescence microscopy course for medical students
2005	Tutor for first and second year medical students, University of Bern

Publications

1. Nischang M, Baenziger S, Hofer U, Schlaepfer E, Gers-Huber G, Regenass S, Sutmuller R, Speck RF. *Modeling antiretroviral therapy in HIV-infected humanized mice.* In preparation.
2. Audigé A*, Hofer U*, Beq S, Assouline B, Morre M, Dittmer U, Speck RF. *Combined IFN-alpha/IL-7 immunotherapy in mice with chronic viral infections.* In preparation.
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* equal contribution

Presentations

2010 Poster: *HIV pathogenesis in vivo.* Gordon research conference, Immunochemistry and Immunobiology, Les Diablerets, Switzerland

2010 Poster: *Defective clearance of translocated bacterial products promotes HIV pathogenesis*. Keystone Symposia, HIV Pathogenesis, Santa Fe, USA

2009 Poster: *Rectal HIV transmission in humanized mice.* Workshop in Fundamental Virology, Fribourg, Switzerland

2008 Talk: *HIV mouse models*. Invited seminar, University Hospital Essen, Germany

2008 Poster: *Humanized RAG2-/-γc-/- mice are resistant to rectal HIV transmission*. Keystone Symposia, HIV Pathogenesis, Banff, Canada

Review Activities

Ad hoc manuscript referee: Current HIV Research, PLoS One

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